PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

WARDER C. ALLEE
Managing Editor

EDITORIAL BOARD

J. H. BODINE, University of Iowa
C. M. CHILD, Stanford University
M. F. GUYER, University of Wisconsin
L. V. HEILBRUNN, University of Pennsylvania
E. E. JUST, Howard University
F. R. LILLIE, University of Chicago
R. S. LILLIE, University of Chicago
W. W. SWINGLE, Princeton University
C. V. TAYLOR, Stanford University
B. H. WILLIER, University of Rochester

VOLUME XI
1938

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO ILLINOIS

THE CAMBRIDGE UNIVERSITY PRESS, LONDON
THE MARUZEN COMPANY, LIMITED, TOKYO
THE COMMERCIAL PRESS, LIMITED, SHANGHAI

PUBLISHED
JANUARY, APRIL, JULY, OCTOBER, 1938

COMPOSED AND PRINTED BY THE UNIVERSITY OF CHICAGO
PRESS, CHICAGO, ILLINOIS, U.S.A.

54
3

Medical

CONTENTS OF VOLUME XI

NUMBER 1

The Relation between Temperature, Salts, Hydrogen Ion Concentration, and Frequency of Ingestion of Food by *Amoeba* . . . . . *S. O. Mast and R. A. Fennell* 1

The Effect of Chemicals and Temperature on Reversal in Ciliary Action in *Paramecium* *Joseph F. Oliphant* 19

The Influence of Certain Plant Hormones on Growth of Protozoa . *Alfred M. Elliott* 31

Some Effects of Hypertonic Solutions upon Development and Oxygen Consumption *Arthur Louis Schipper* 40

A Study of the Mechanism of Cyanide Inhibition. I. Effect of Concentration on the Egg of *Melanoplus differentialis* . . . . *W. A. Robbie, E. J. Boell, and J. H. Bodine* 54

Comparison of Quantitative Precipitin Techniques as Influenced by Injection Procedures *Harold R. Wolfe and Joseph G. Baier, Jr.* 63

The Influence of the Frequency of Stimulation on the Slow and the Fast Contraction in Crustacean Muscle . . . . . *C. A. G. Wiersma and A. Van Harreveld* 75

The Response to Mechanical Shock by the Cercariae of *Bucephalus elegans* *Harry T. Folger and Lloyd E. Alexander* 82

The Nutritional Requirements of *Blattela germanica* . . . . . *C. M. McCay* 89

NUMBER 2

Annotated Bibliography of the Scientific Publications of Professor Charles Manning Child *Libbie H. Hyman and C. D. Van Cleave* 105

The Fragmentation of *Nais paraguayensis* . . . . . *Libbie H. Hyman* 126

The Effect of Planarian Extracts and Exudates upon Head Regeneration in *Euplanaria dorotocephala* . . . . . *J. William Buchanan* 144

The Microscopic Anatomy of Twins and Double Monsters of *Fundulus heteroclitus* *Marie A. Hinrichs* 155

The Influence of Thyroxin on Eye Formation in *Phagocata gracilis* (Haldeman) *Bernal R. Weimer, Charles W. Phillips, and Dagney M. Andersen* 158

The Effect of Dead Optic Vesicle upon Explants of Prospective Ectoderm *C. D. Van Cleave* 168

Oxygen as a Controlling Factor in the Regeneration of Tubularia . . . *L. G. Barth* 179

Differential Gradient Functions in the Feather Germ . . . . *Richard M. Fraps* 187

Single and Combined Effects of Cyanide and Methylene Blue on Reconstitution in *Euplanaria dorotocephala* . . . . . *Olin Rulon* 202

Studies on Heteroplastic Transplantation in Triclads. I. Cephalic Grafts between *Euplanaria dorotocephala* and *E. tigrina* . . . . . *James A. Miller* 214

NUMBER 3

Modifications of Development in Relation to Differential Susceptibility of the Bruchid (Coleoptera) Egg to KCN during Different Metabolic Phases . . *Alfred Brauer* 249

Ultra-Violet Radiation of Grasshopper Eggs (Orthoptera) *O. Malcolm Ray* and *Joseph Hall Bodine* 267

The Rate of Oxygen Consumption of Frogs during Embryonic Development and Growth *Meyer Atlas* 278

Light Intensity and Melanophore Response in a Cyprinid Fish *Ragnar Nathanael Danielson* 292

The Similarity between the Hypophyseal Chromatophorotropic Hormone and the Chromatophorotropic Hormone of the Crustacean Eyestalk . *A. A. Abramowitz* 299

On the Motor and Inhibitory Innervation of the Legs of Brachyuran Decapods *C. A. G. Wiersma and George Marmont* 312

The Olfactory Reactions of *Drosophila melanogaster* Meigen to the Products of Fermenting Banana . . . . . *Melia R. Reed* 317

Race and Conjugation of *Paramecium* . . . . . *Arthur C. Giese* 326

Reproduction of *Paramecium* in Old Culture Medium *W. H. Johnson and Garrett Hardin* 333

Studies on the Physiology of Amoeba. II. The Allelocatalytic Effect in Amoeba Cultures Free of Bacteria . . . . . *Karl Reich* 347

NUMBER 4

Erratum v

The Effect of Substances Produced by *Chilomonas paramecium* on Rate of Reproduction *S. O. Mast and D. M. Pace* 359

Fat Release in Amoeba after Irradiation . *L. V. Heilbrunn and Kathryn Daugherty* 383

An Experimental Study of Clot Formation in the Perivisceral Fluid of *Arbacia* *James A. Donnellon* 389

Abundant Feeding Followed by Restricted Feeding and Longevity in *Daphnia* *H. Howard Dunham* 399

Studies in Mass Physiology: Effect of Numbers upon the Oxygen Consumption and Locomotor Activity of *Carassius auratus* . . . . . *Arthur Shlaifer* 408

Fetal Respiratory Movements in the Cat *W. F. Windle, M. Monnier, and A. G. Steele* 425

Physiology of Development of the Feather. II. General Principles of Development with Special Reference to the After-Feather . . . . *Frank R. Lillie and Mary Juhn* 434

Index to Volume XI 451

lume XI
MEDICAL LIBRARY
JAN 24 1938
Number 1

Physiological Zoölogy

JANUARY, 1938

The Relation between Temperature, Salts, Hydrogen Ion Concentration, and Frequency of Ingestion of Food by Amoeba - *S. O. Mast and R. A. Fennell* 1

The Effect of Chemicals and Temperature on Reversal in Ciliary Action in *Paramecium* - - - - - - - - - - - - - *Joseph F. Oliphant* 19

The Influence of Certain Plant Hormones on Growth of Protozoa *Alfred M. Elliott* 31

Some Effects of Hypertonic Solutions upon Development and Oxygen Consumption *Arthur Louis Schipper* 40

A Study of the Mechanism of Cyanide Inhibition: I. Effect of Concentration on the Egg of *Melanoplus differentialis* - *W. A. Robbie, E. J. Boell, and J. H. Bodine* 54

Comparison of Quantitative Precipitin Techniques as Influenced by Injection Procedures - - - - - - - *Harold R. Wolfe and Joseph G. Baier, Jr.* 63

The Influence of the Frequency of Stimulation on the Slow and the Fast Contraction in Crustacean Muscle - - - *C. A. G. Wiersma and A. Van Harreveld* 75

The Response to Mechanical Shock by the Cercariae of *Bucephalus elegans* *Harry T. Folger and Lloyd E. Alexander* 82

The Nutritional Requirements of *Blattela germanica* - - - *C. M. McCay* 89

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO, ILLINOIS, U.S.A.

PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

WARDER C. ALLEE
Managing Editor

EDITORIAL BOARD

J. H. BODINE, *University of Iowa*
C. M. CHILD, *Stanford University*
M. F. GUYER, *University of Wisconsin*
L. V. HEILBRUNN, *University of Pennsylvania*
E. E. JUST, *Howard University*
F. R. LILLIE, *University of Chicago*
R. S. LILLIE, *University of Chicago*
W. W. SWINGLE, *Princeton University*
B. H. WILLIER, *University of Rochester*
C. V. TAYLOR, *Stanford University*

Physiological Zoölogy is published quarterly by the University of Chicago at the University Press, 5750 Ellis Avenue, Chicago, Ill. The subscription price is $7.50 a year; the price of single copies is $2.25. Orders for service of less than a half-year will be charged at the single-copy rate. Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Hayti, Uruguay, Paraguay, Hawaiian Islands, Philippine Islands, Guam, Samoan Islands, Balearic Islands, Spain, and Venezuela. Postage is charged extra as follows: for Canada and Newfoundland, 15 cents on annual subscriptions (total $7.65), on single copies 4 cents (total $2.29); for all other countries in the Postal Union, 40 cents on annual subscriptions (total $7.90), on single copies 10 cents (total $2.35). Patrons are requested to make all remittances payable to The University of Chicago Press in postal or express money orders or bank drafts.

The following are authorized agents:

For the British Empire, except North America, India, and Australasia: The Cambridge University Press, Fetter Lane, London, E.C. 4, England. Prices of yearly subscriptions and of single copies may be had on application.

For Japan: The Maruzen Company, Ltd., Tokyo.

For China: The Commercial Press, Ltd., 211 Honan Road, Shanghai. Yearly subscriptions, $7.50; single copies, $2.25, or their equivalents in Chinese money. Postage extra on yearly subscriptions 40 cents, on single copies 10 cents.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to W. C. Allee, Managing Editor of PHYSIOLOGICAL ZOÖLOGY, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y.

Applications for permission to quote from this journal should be addressed to The University of Chicago Press, and will be freely granted.

Entered as second-class matter October 27, 1928, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

[PRINTED IN U.S.A.]

Physiological Zoölogy

Vol. XI JANUARY 1938 *No. 1*

THE RELATION BETWEEN TEMPERATURE, SALTS, HYDROGEN ION CONCENTRATION, AND FREQUENCY OF INGESTION OF FOOD BY AMOEBA[1]

(Eight figures)

S. O. MAST AND R. A. FENNELL
Zoölogical Laboratory, Johns Hopkins University

INTRODUCTION

IT IS well known that under some conditions amoebae do not feed, although the culture medium contains an abundance of food, and that under other conditions some feed and others do not. This shows that feeding in amoebae depends upon the condition of the organisms and the condition of the environment. However, only a few detailed observations have been made concerning the determining factors.

Schaeffer (1916) maintains that the most important single factor in inducing feeding in amoebae is movement of the food, but that the character of the food particles taken in is also involved. He says that, in general, soluble particles are more readily taken than insoluble ones.

Mast and Hahnert (1935) came to the following conclusion:

> *Amoeba proteus* does not feed when it is not attached to the substratum, therefore anything which facilitates attachment facilitates feeding. It has been repeatedly observed that feeding increased greatly if amoebae were transferred from a solution in which attachment was weak, to one in which it was strong, e.g., fresh Chalkley solution. It was repeatedly observed that feeding increased if the amoebae were agitated. This was particularly marked in specimens transferred from ordinary culture dishes to slides. Starvation facilitates feeding, but not if it continues until the amoebae no longer attach firmly. Specimens kept without food 5 to 8 days usually do not feed again no matter how they are treated, although they live 10 days or longer.

Aside from these facts, little is known concerning the factors which determine feeding in *Amoeba*. More is known, however, concerning these factors in other protozoa.

Schaeffer (1910) made a very thorough study of feeding in *Stentor caeruleus*. He maintains that hunger accelerates frequency of ingestion, that satiety retards it, and that *Stentor* takes some organisms more readily than others.

[1] We are indebted to the Marine Biological Laboratory at Woods Hole, Massachusetts, for facilities used in making some of the experiments considered below.

Metalnikow (1912) maintains that low temperatures, high temperatures, weak alkaline solutions, and old culture solutions retard frequency of ingestion of particles by paramecia, and that moderate temperatures, weak acid solutions, alcohols, and arsenious compounds accelerate it. He found also that paramecia ingests digestible substances more readily than indigestible substances.

MATERIALS AND METHODS

Kaulbaum's "purest" chemicals and triple (Pyrex glass) distilled water were used in all solutions. The salts were carefully dried and weighed, and in some instances purified by recrystallization. The concentration of the hydroxide solutions used was ascertained by titrating against a known amount of potassium acid phthalate ($KHC_8H_4O_4$), as outlined by Clark (1928). The solutions were stored in Pyrex glass flasks, which contained an outlet with a stopcock and an inlet with a soda-lime tube attached. The concentration of $MgCl_2$ and $CaCl_2$ was ascertained by the method described by Blasdale (1931). The concentration of the phosphates used was ascertained by the molybdenum-blue method of Deniges, as modified by Farber and Youngburg (1932). Calcium chloride was substituted for $CaH_4(PO_4)_2$ in solutions with low hydrogen ion concentrations, so as to avoid precipitation of the calcium.

The temperature of the solutions in which feeding was tested was controlled by means of a temperature bath, designed by Pitts (1932). This bath consists of a small brass box which fits on the stage of the microscope and contains a flat-bottom glass dish surrounded by a rubber diaphragm to prevent leakage of water. The glass dish was made as follows: A 50-cc. Pyrex glass beaker was painted inside and outside with paraffin; then a line in the paraffin 2 mm. wide was made around the beaker 2.5 cm. from the bottom, after which it was immersed in hydrofluoric acid and left 24 hours. During this time the acid dissolved the glass which was not covered by paraffin, separating the bottom from the top. The rough edges of the bottom were ground down and polished. The temperature in the glass dish was adjusted by regulating the flow of water of the desired temperature through the bath. The hydrogen ion concentration of the solutions was measured electrically.

Chilomonas paramecium cultured in sterile glucose-peptone solution (1.5 g. glucose and 3.5 g. peptone in 1,000 cc. water) was used as food in all experiments made. Sterile cultures were obtained by a method similar to that which was perfected by Hargitt and Fray (1917) and Phillips (1922). Twenty-five chilomonads were put into 0.4 cc. of glucose-peptone solution on a sterile depression slide in a sterile Petri dish. Then the dish was covered and left 5 minutes. The chilomonads were then transferred with a sterile pipet (plugged with sterile cotton at the bulb end) to the same amount of solution on another depression slide in another Petri dish, which was covered and left 5 minutes. This process was then repeated thirteen times, after which one of the chilomonads was transferred to each of fourteen sterile 125 cc. flasks, each of which contained 50 cc. of sterile glucose-peptone solution.

The flasks were left from 5 to 7 days; then one of the sterile flasks was used to inoculate additional flasks, each containing 50 cc. of sterile glucose-peptone solution. The freshly inoculated flasks were left 48 hours, that is, until chilomonads were abundant. Then the chilomonads in three of these flasks were transferred, as follows, to the solution used in the tests on feeding: Fifteen cubic centimeters of the solution in one of the flasks was put into each of two centrifuge tubes and centrifuged at a moderate speed for

5 minutes, after which all but 1 cc. of the solution (i.e., the solution which contained the chilomonads) was poured off. Then 14 cc. of the solution from the flask was added to each test tube. This was repeated until all the solution in the three flasks was centrifuged. Then 14 cc. of the solution to be used for the test on feeding was added to each tube and centrifuged, after which all but 1 cc. at the bottom of the tube was poured off. This procedure was repeated three times. During the successive transfers the glucose-peptone in which chilomonads were cultured was diluted about 40,000 times. Chilomonads were prepared in this way for each test on feeding, as described below.

The amoebae used were cultured in Chalkley solution (see p. 4) containing rice and wheat. Three weeks after inoculation the best culture was selected; and from this 250 amoebae[2] of the same size and appearance were taken, put into a small Pyrex glass dish which contained 5 cc. distilled water, and left 10 minutes. Then they were transferred through the same amount of distilled water three more times, but after the last transfer they were left for 24 hours instead of 10 minutes. At this time each amoeba had three or four long, slender pseudopods and little, if any, food (no fat or starch), and most of them were not attached or only loosely attached to the substratum. From these 250 amoebae, 100 as nearly as possible the same in size, structure, and form were selected and put into a small beaker which contained 5 cc. of the solution to be used in the test on feeding, and left 5 minutes. Then they were transferred, in the same way, through the same amount of solution three more times, after which they were transferred to the same amount of solution in the small glass dish in the Pitts temperature bath, which was now covered, sealed with vaseline, and left undisturbed for 15 minutes for adjustment to the solution. Then the cover of the dish was removed, and 2 cc. of the solution containing chilomonads prepared for tests on feeding were added, after which the dish was again covered and sealed. Fifteen minutes after adding the chilomonads to the bath, 10 amoebae were taken out, put on a glass slide between parallel ridges of vaseline, and covered with a cover slip. Then some of the solution was removed with filter paper so as to flatten the amoebae; then the chilomonads in each amoeba were counted, and the mean calculated and recorded. This was repeated 30, 60, and 120 minutes after the chilomonads had been in the Pitts bath. The results obtained are considered in the following pages.

THE RELATION BETWEEN ABUNDANCE OF FOOD AND FREQUENCY OF INGESTION BY *Amoeba proteus*

Metalnikow (1912) maintains that in paramecia the rate of feeding is, within wide limits, independent of the abundance of food in the culture solution; but Fenn (1921) maintains that in leucocytes the number of particles ingested by them is directly proportional to the number of particles in suspension.

One hundred selected amoebae in Chalkley solution were put into each of two Pitts temperature baths, adjusted to 24° C. Then about 925,000 chilomonads were added to one and 1,850,000 to the other, after which Chalkley solution was added until the volume in both was 3 cc.; then the number of chilomonads ingested by the amoebae was ascertained, as described above.

The results are given in Table 1. These results show that the number of chilomonads

[2] Each time that amoebae were removed from a culture, the solution taken out with them was replaced with distilled water. Owing to this, the hydrogen ion concentration in the cultures remained nearly constant

ingested in 15- and 30-minute periods was slightly higher in the solution which contains the greater number of chilomonads, but that the number ingested in 60 and 120 minutes was nearly the same in both solutions. This demonstrates that there can be great variation in the abundance of chilomonads in the solution without appreciably affecting the number of chilomonads ingested by the amoebae, if they are left half an hour or longer.

THE RELATION BETWEEN TEMPERATURE AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus and Amoeba dubia*

Observations were made on ingestion of chilomonads by *A. proteus* in Chalkley solution,[3] and by *A. dubia* in Hahnert (1932) solution, at temperatures of 5°, 10°, 15°, 20°, 25°, 30°, and 35° C. These observations were made as described in the preceding pages.

TABLE 1

THE RELATION BETWEEN ABUNDANCE OF FOOD AND FREQUENCY OF INGESTION BY *Amoeba proteus* (6 TO 10 AMOEBAE USED IN EACH TEST)

Number of Chilomonads per Cubic Centimeter	Average Number of Chilomonads Ingested by 6–10 Amoebae				Numbers Ingested per Individual in 120 Min.	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
600,000	12	41	56	80	63	96
300,000	9	36	57	81	64	98

Three sets of observations were made, two on *A. proteus* and one on *A. dubia*. The results obtained in the first set of observations on *A. proteus* and those obtained in observations on *A. dubia* are presented in Table 2 and Figure 1. Figure 1 shows, in these two sets of observations, that, as the temperature increased, the frequency of ingestion of chilomonads by *A. proteus* and by *A. dubia* increased to a maximum at about 26° and then decreased to zero at 40°, but that the number ingested by *A. proteus* in 120 minutes decreased to a median minimum at 30°, and to a second maximum at 35°, before it decreased to zero. Similar results were obtained in the second set of observations on *A. proteus*, but there was no median minimum. The median minimum obtained in the first set is therefore probably not significant. The results as a whole, show that frequency of ingestion in amoebae is closely correlated with temperature.

Table 2 shows that at 25° an average of 63 chilomonads per amoeba were ingested in 120 minutes and that, of these, 26 were ingested during the first 15 minutes, 10 during the next 15 minutes, 20 during the next 30 minutes, and only 7 during the last 60 minutes. The frequency of ingestion therefore decreased from 1.73 chilomonads per minute during the first 15 minutes, when the amoebae contained from 0 to 25 chilomonads each, to only 0.1 chilomonad per minute during the last 60 minutes, when they contained from 56 to 63 each. Table 2 shows also that similar results were obtained at 5°, 10°, 15°, 20°, 25°, and 30° C. Similar results were also obtained in the second set of

[3] Chalkley solution: 80 mg. NaCl, 4 mg. $NaHCO_3$, 4 mg. KCl, 4 mg. $CaCl_2$, 2 mg. $CaH_4(PO_4)_2$, 2 mg. $Mg_3(PO_4)_2\,4H_2O$, and 1,000 cc. distilled water. Hahnert solution: 4 mg. $NaHCO_3$, 4 mg. KCl, 4 mg $CaCl_2$, 2 mg. $CaH_4(PO_4)_2$, 2 mg. $Mg_3(PO_4)_24H_2O$, and 1,000 cc. distilled water.

tests with *A. proteus* and in the set of tests with *A. dubia*. It is consequently evident that the frequency of ingestion varies inversely with the amount of food in the amoebae.

TABLE 2

THE RELATION BETWEEN TEMPERATURE AND THE FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus*

Temperature in Degrees C.	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers Ingested per Individual in 120 Min.	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
5	0.8	0.6	6	9	4	16
10	1.1	7	12	13	6	25
15	2	10	18	43	32	56
20	7	19	37	50	38	60
25	26	36	56	63	46	72
30	25	43	50	51	42	65
35	13	10	43	68	46	86
40	0	0	0	0	0	0

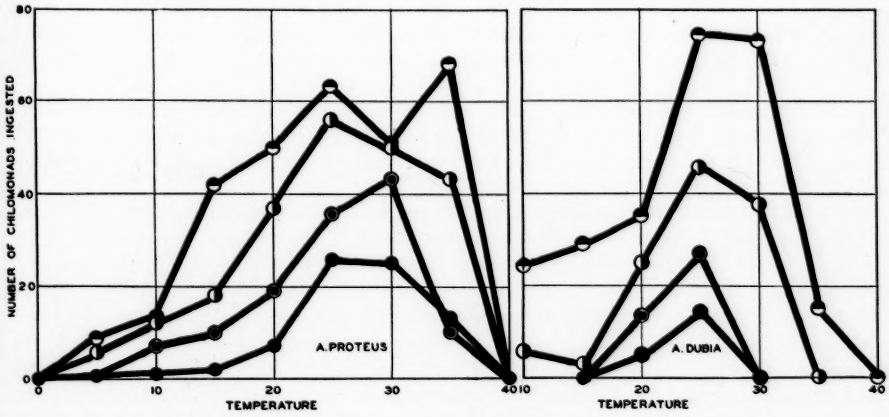


FIG. 1.—The relation between temperaure and frequency of ingestion of chilomonads by *Amoeba*. ●, number of chilomonads ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals. The extent of individual variation in *A. proteus* is presented in Table 2. The variation in *A. dubia* was practically the same.

Table 2 shows that at 35° an average of 13 chilomonads were ingested during the first 15 minutes, none during the next 15 minutes, 33 during the next 30 minutes, and 25 during the last 60 minutes, i.e., that the frequency of ingestion decreased from 0.86 chilomonads per minute during the first 15 minutes to zero during the second, then in-

creased to 1.10 during the next 30 minutes, and then decreased to 0.41 during the last 60 minutes. These results indicate that the frequency of ingestion at 35° is not closely correlated with the number of chilomonads in the amoebae. This is probably due to variations in attachment to the substratum, for the amoebae were fairly firmly attached during the first 15 minutes, when the frequency of ingestion was high, and only loosely during the second 15 minutes, when it was low, but were again fairly firmly attached after that, when it was again high. This indicates that the frequency of ingestion varies directly with the degree of attachment to the substratum, and that the increase in frequency after exposure of 30 minutes at 35° is due to adjustment, resulting in increase in the degree of attachment.

Mast and Prosser (1932) maintain that, as the temperature increases, the rate of locomotion in *A. proteus* increases to a maximum at about 25°, then decreases to a median minimum at about 28°, then increases to a second maximum at about 30°, and then rapidly decreases to zero. Schwitalla (1925) obtained results in accord with these views. This indicates that rate of locomotion is correlated with temperature in much the same way as frequency of ingestion.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTANING SODIUM AS THE ONLY METALLIC CATION AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The experiments described in this section were made with two series of solutions which consisted of NaH_2PO_4 and NaOH in water. Each of the two series was prepared by mixing NaH_2PO_4 of a given molecular concentration with NaOH of the same molecular concentration in such proportions as to produce a series of hydrogen ion concentrations from pH 4.4 to 8.3. The concentration of the sodium was consequently the same in all the solutions in each of the two series. It was 0.002 N in one and 0.005 N in the other. To obtain hydrogen ion concentrations of pH 4.4, a small amount of 0.1 M HCl was added. Observations were made as described in the preceding pages. The results obtained are presented in Table 3 and Figure 2.

Figure 2 shows that, as the hydrogen ion concentration decreased, the frequency of ingestion of chilomonads by amoebae increased from zero at pH 4.4, nearly to a secondary maximum at pH 5.0, then remained nearly constant to pH 5.5, then increased to a primary maximum between pH 6.0 and 6.5, then decreased to zero at pH 7.7–8.3. It shows that the rate of ingestion in the stronger solution of sodium was consistently much higher than in the weaker solution and that there was somewhat of a median minimum near pH 6.5 in both concentrations.

Figure 2 indicates that frequency of ingestion is more closely correlated with the hydrogen ion concentration in the stronger than in the weaker solution, and that the maximum acidity at which ingestion occurred was the same in both concentrations but that the maximum alkalinity[4] at which it occurred was higher in the weaker than in the stronger solution.

Table 3 columns 6 and 7, show that there was marked individual variation in the frequency of ingestion in the weaker solution but that the averages are consistent throughout. The variation in the stronger solution was essentially the same as in the weaker.

[4] "Maximum alkalinity" is defined as the lowest hydrogen ion concentration in which ingestion occurs; and "maximum acidity," as the highest in which it occurs.

TABLE 3

THE RELATION BETWEEN FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus* AND HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING SODIUM AS THE ONLY METALLIC CATION (CONCENTRATION OF SODIUM, 0.002 N)

Hydrogen Ion Concentration in pH Units	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers Ingested per Individual in 120 Minutes	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
4.4	0	0	0	0	0	0
4.7	16	31	34	43	32	56
5.0	22	34	37	53	39	68
5.5	21	26	36	52	35	74
6.0	23	34	44	51	23	78
6.3	20	29	47	53	35	72
6.6	18	22	33	67	53	76
7.0	19	23	40	47	40	58
7.4	21	38	49	49	20	110
7.8	18	29	42	50	28	70
8.1	17	19	32	44	28	65
8.4	0	0	0	0	0	0

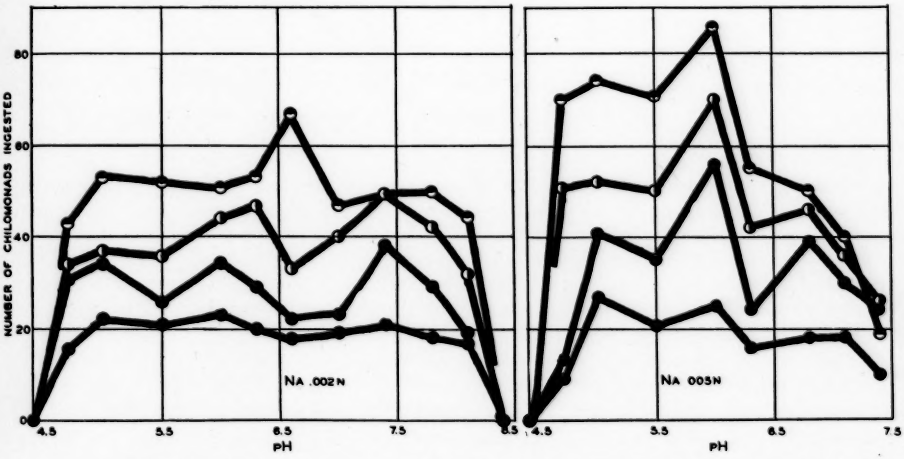


FIG. 2.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing sodium as the only metallic cation. ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals. Lowest and highest numbers of chilomonads ingested per individual in 120 minutes is 32 and 56, respectively, at pH 4.7; 53 and 76 at pH 6.6, the optimum hydrogen ion concentration. Similar individual variations occurred in all the other tests.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING POTASSIUM AS THE ONLY METALLIC CATION AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The methods used in making observations on the effect of potassium on frequency of ingestion of food by amoebae were the same as those used in observations on the effect of sodium. The results are presented in Figure 3.

Figure 3 shows that as the hydrogen ion concentration increased the frequency of ingestion of chilomonads increased from zero at pH 4.4 to a maximum at pH 5.0 and then decreased nearly to zero, but that it decreased much more gradually in the weaker than in the stronger solution and that the maximum alkalinity was considerably higher

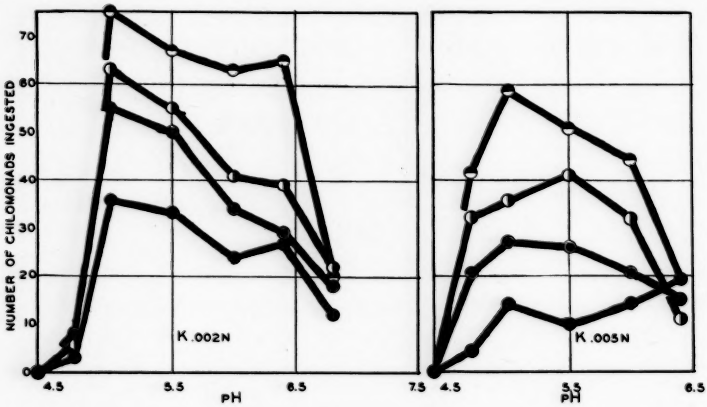


FIG. 3.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing potassium as the only metallic cation. ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals. The individual variation was similar to that in solutions containing sodium in place of potassium.

in the former than in the latter. It indicates that frequency of ingestion of food is more closely correlated with hydrogen ion concentration in the stronger than in the weaker solutions of potassium, as it is in solutions of sodium.

It will be seen by comparing Figure 3 with Figure 2 that the results obtained with potassium are similar to those obtained with sodium—that in both, the alkaline limit for ingestion is more acid in the stronger than in the weaker solution, but that at the optimum for frequency of ingestion, the solutions which contain potassium are more acid than those which contain sodium, and that, as the concentration increased, frequency of ingestion decreased in the former and increased in the latter.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING CALCIUM AS THE ONLY METALLIC CATION AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The methods used in making observations on the effect of calcium on frequency of ingestion of food by amoebae were the same as those used in observations on the effect

of sodium, except that $CaCl_2$ was substituted for $CaH_4(PO_4)_2$ in all solutions with hydrogen ion concentrations lower than pH 6.4, because calcium is precipitated in these solutions if phosphates are present, and that the concentration of calcium in the two series of solutions was 0.001 M and 0.0025 M instead of 0.002 M and 0.005 M, and that the observations (owing to increase in hydrogen ion concentration in the alkaline solutions) were continued for only 30 minutes in place of 120 minutes. The results are presented in Figure 4.

Figure 4 shows that, as the hydrogen ion concentration decreased, the frequency of ingestion of chilomonads gradually increased to a maximum approximately at pH 6.0, then remained constant until pH 7.0 was reached, then decreased until the close of the experiment at pH 8.0 but not to zero.

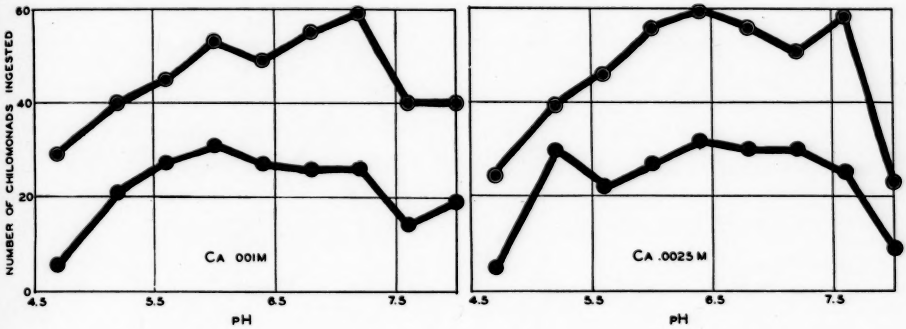


FIG. 4.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing calcium as the only metallic cation. Ordinates, number of chilomonads ingested; abscissae, hydrogen ion concentration in pH units; ●, number ingested in 15 minutes; ◉, 30 minutes. Each point on the curves represents the average for 10 individuals.

Figure 4 also shows that frequency of ingestion of chilomonads was about the same in the weaker and the stronger solutions of calcium, but that the frequency of ingestion at the alkaline limit was much higher in the weaker than in the stronger solution.

It can be seen by comparing Figures 2 and 3 with Figure 4 that the effect of hydrogen ion concentration on frequency of ingestion in calcium solutions differs greatly from that in either sodium or potassium solutions, in that the frequency of ingestion is much higher in the acid and much lower in the alkaline range in the former than in the latter.

The results indicate that the effect of calcium differs qualitatively from either that of sodium or that of potassium. Pitts and Mast (1934) reached the same conclusion in their study on the relations between hydrogen ion concentration in solutions of single salts and rate of locomotion of amoebae.

THE RELATION BETWEEN HYDROGEN ION CONCENTRATION IN SOLUTIONS CONTAINING SODIUM AND CALCIUM IN DIFFERENT PROPORTIONS AND FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus*

The observations described in this section were made with two series of solutions which contained NaH_2PO_4, NaOH, and $CaCl_2$ in water. In both series, 0.005 M NaH_2-

PO_4 and 0.005 M NaOH were mixed in various proportions so as to produce solutions which differed in hydrogen ion concentration as described above. To each solution of one series enough $CaCl_2$ was added to make the concentration of calcium 0.001 M; and to each of the other series, enough to make the concentration of calcium 0.003 M. The calcium and the sodium concentrations of all the solutions were therefore the same in each series. The observations were made as described in the preceding pages. The results obtained are presented in Figure 5.

Figure 5 shows that, as the hydrogen ion concentration decreased, frequency of ingestion increased to a maximum at about pH 6.2, then decreased to a median mini-

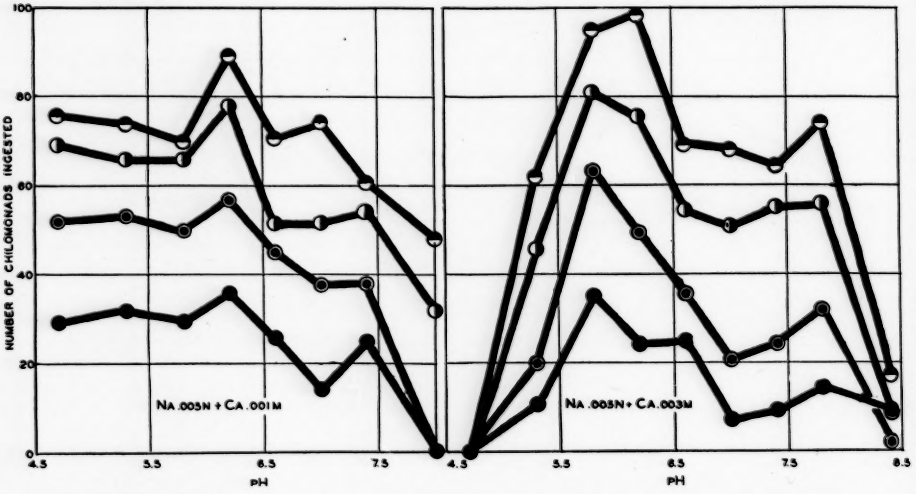


FIG. 5.—The relation between frequency of ingestion of chilomonads by *A. proteus* and hydrogen ion concentration in solutions containing sodium and calcium. *A*, concentration of sodium (0.005 N), calcium (0.001 M); *B*, sodium (0.005 N), calcium (0.003 M); ordinates, numbers of chilomonads ingested; abscissae, hydrogen ion concentration in pH units; ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals.

mum at approximately pH 7, and then decreased to zero at about pH 8.5. The figure shows that the median minimum is much more marked in the solution containing the higher concentration of calcium than that containing the lower concentration, and that the frequency of ingestion, in the alkaline range, was relatively much higher in the former than in the latter.

By comparing Figure 5 with Figure 2, it will be seen that the addition of calcium 0.001 M to solutions containing sodium 0.005 M had little, if any effect on the frequency of ingestion in the stronger acid solutions (pH 4.7–5.7) but that it caused considerable increase in frequency in the weaker acid and the alkaline solutions (pH 6.2–8.0) with a shift in the maximum from pH 6.0 to 6.2, and that addition of calcium 0.003 M

caused greater increase in frequency in the weaker acid and the alkaline solutions (pH 5.7–8.2) and marked decrease in the stronger acid solutions (pH 4.7–5.5).

The results obtained in this study are similar to those obtained by Pitts and Mast (1934), who found that, as the hydrogen ion concentration increased, in solutions containing 0.005 N sodium and 0.001 M calcium, rate of locomotion increased from zero at pH 4.5 to a maximum at pH 5.9, then decreased to a median minimum at pH 7.0, then increased to a second maximum at pH 7.5 then, decreased again. Hopkins (1928), using modified Ringer solution, and Mast and Prosser (1932), using a solution containing 0.005 N sodium and 0.002 M calcium, obtained similar results. This supports the conclusion reached in the preceding section, that frequency of ingestion of food and rate of locomotion are closely correlated.

TABLE 4

THE EFFECT OF DIFFERENT CONCENTRATIONS OF NaCl ON FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus*

Molar Concentration of NaCl	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers Ingested per Individual in 120 Minutes	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
0.0003	4	26	35	58	35	73
0.0008	37	72	106	185	154	250
0.002	29	55	93	118	81	163
0.005	29	57	90	110	56	225
0.008	29	45	90	100	62	165
0.01	19	43	86	86	65	120
0.02	25	38	44	55	40	82
0.03	4	7	8	10	0	30

THE RELATION BETWEEN NaCl, KCl, $MgCl_2$, AND $CaCl_2$ IN DIFFERENT CONCENTRATIONS AND THE FREQUENCY OF INGESTION OF FOOD BY *Amoeba proteus* and *A. dubia*

The experiments described in this section were made with different concentrations of the chlorides of sodium, potassium, magnesium, and calcium. The observations were made as described in the preceding pages. The results are presented in Table 4 and Figures 6 and 7.

Figure 6 and Table 4 show that, as the concentration of NaCl increased from 0.0003 M, frequency of ingestion of chilomonads very rapidly increased to a maximum at 0.0008 M, then very gradually decreased nearly to zero at 0.03 M, and that at the optimum concentration an average of 185 chilomonads were ingested by each amoeba in 120 minutes.

Figure 6 also shows that the relation between frequency of ingestion and concentration in solutions of KCl is similar to what it is in solutions of NaCl but that the range of concentrations over which ingestion occurs is very much shorter in the former than it is in the latter, and that frequency of ingestion is consistently lower throughout the range, the average maximum frequency being 185 in 120 minutes in NaCl and only 90

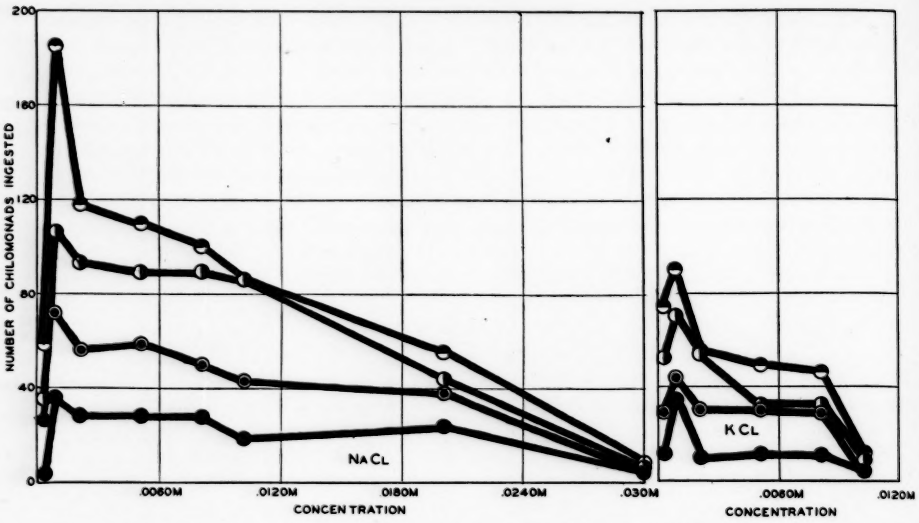


FIG. 6.—The effect of concentrations of salts on frequency of ingestion of chilomonads by *A. proteus*. *A*, NaCl; *B*, KCl; ordinates, number of chilomonads ingested; abscissae, molar concentration; ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals.

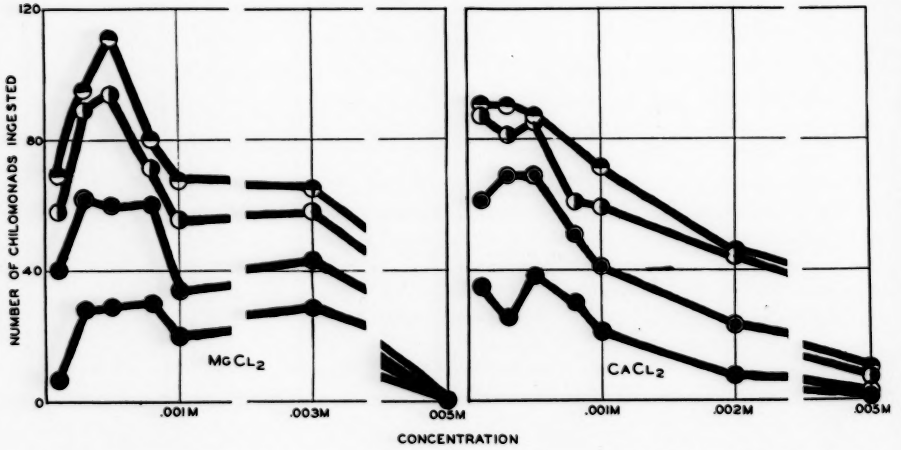


FIG. 7.—The effect of different concentrations of salts on frequency of ingestion of chilomonads by *A. proteus*. *A*, $MgCl_2$; *B*, $CaCl_2$; ordinates, number of chilomonads ingested; abscissae, molar concentration; ●, number ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes.

in KCl. The results consequently show that potassium solutions are much less favorable for ingestion than sodium solutions.

These results agree with those obtained by Mast and Prosser (1932) in their study of amoeboid locomotion in different concentrations of KCl. They found that it is extremely difficult to obtain active amoebae in any concentration of KCl, except 0.001 N, and that in this concentration locomotion continued only a short time. Heilbrunn and Daugherty (1932) contend that potassium has strong liquefying action on the plasmagel of amoebae and that it is consequently highly toxic.

Figure 7 shows that, as the concentration of $MgCl_2$ increased from 0.0001 M, frequency of ingestion increased to a maximum at 0.0005 M and then decreased to zero at 0.005 M. It also shows that the relation between frequency of ingestion and concentration in solutions of $CaCl_2$ is similar to what it is in solutions of $MgCl_2$, but that in the lowest concentration used, it is much higher in $CaCl_2$ than in $MgCl_2$ and at optimum concentration somewhat higher in $MgCl_2$ than in $CaCl_2$.

The results presented in Figures 6 and 7 show that the optimum concentration for ingestion of chilomonads by amoebae is at 0.0008 M for NaCl and KCl, at 0.0005 for $MgCl_2$, and probably somewhat lower for $CaCl_2$ The results show also that the maximum frequency of ingestion is highest in NaCl solutions (185), lower in $MgCl_2$ (111), still lower in $CaCl_2$ (91), and lowest in KCl (90); that the maximum concentration in which ingestion occurs is highest in NaCl (0.03 M), much lower in KCl (0.01 M), and still lower in $MgCl_2$ and $CaCl_2$ (0.005 M); and that the minimum concentration in which ingestion occurs is much lower in $MgCl_2$ and $CaCl_2$ (0.0001 M), than in NaCl and KCl (0.0003 M). They show, moreover, that the range of concentration over which ingestion occurs is much greater in NaCl (0.03 M) than in KCl (0.01 M), and much greater in KCl than in $MgCl_2$ and $CaCl_2$ (0.005 M), i.e., that it is more than six times as great in NaCl as in $MgCl_2$ or $CaCl_2$, and about three times as great as in KCl.

The facts that the frequency of ingestion at optimum concentration is approximately twice as high in NaCl as it is in KCl, $MgCl_2$ or $CaCl_2$, and that the range of concentrations over which ingestion occurs is approximately six times as great in NaCl as it is in $MgCl_2$ or $CaCl_2$ and approximately three times as great as it is in KCl, show that ingestion is not specifically correlated with any of the properties common to all these salts—as, for example, osmosis.

THE RELATION BETWEEN OSMOTIC CONCENTRATION AND FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba proteus* AND *A. dubia*

Observations on frequency of ingestion by *A. proteus* and *A. dubia* were made in lactose solution, Chalkley solution (see p. 4), Hahnert solution, and Hahnert solution containing various quantities of lactose. The results are presented in Table 5 and Figure 8.

Table 5 shows that in *A. dubia* the frequency of ingestion was zero in lactose solutions of all concentrations tested, that it was much higher in Chalkley solution than in Hahnert solution, that addition of lactose to Hahnert solution caused marked increase in frequency of ingestion, and that, as the concentration of lactose increases, the frequency increases to a maximum and then decreases.

Figure 8 shows that the results obtained with *A. proteus* are essentially the same as those obtained with *A. dubia*, but that the frequency of ingestion was very much lower, the maximum in 2 hours being more than 400 for the former and only 99 for the latter.

TABLE 5

THE RELATION BETWEEN FREQUENCY OF INGESTION OF CHILOMONADS BY *Amoeba dubia* AND OSMOTIC PRESSURE

Molar Concentration of Solution Used	Average Number of Chilomonads Ingested by 10 Amoebae				Numbers per Individual Ingested in 120 Minutes	
	15 Min.	30 Min.	60 Min.	120 Min.	Lowest Number	Highest Number
0.0001 M lactose	0	0	0	0	.....	.....
0.0005 M lactose	0	0	0	0	.....	.....
0.001 M lactose	0	0	0	0	.....	.....
0.002 M lactose	0	0	0	0	.....	.....
0.003 M lactose	0	0	0	0	.....	.....
0.035 M lactose	0	0	0	0	.....	.....
Chalkley solution	71	137	196	374	358	396
Hahnert solution	34	72	135	193	172	215
Hahnert solution plus 0.03 M lactose	54	123	171	263	240	325
Hahnert solution plus 0.05 M lactose	60	150	232	336	275	405
Hahnert solution plus 0.07 M lactose	51	68	153	169	100	270

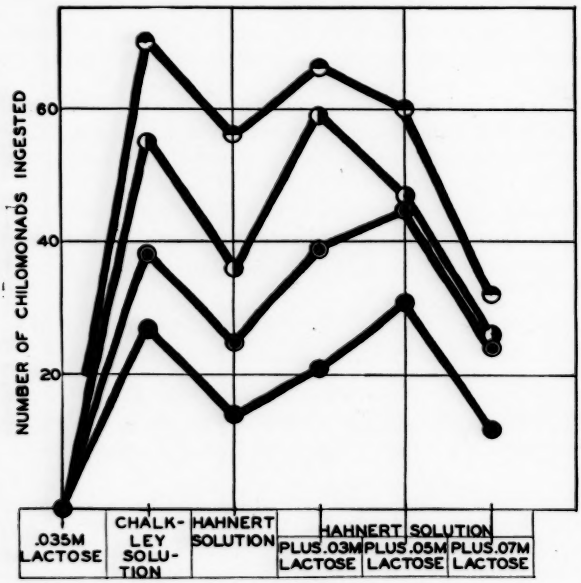


FIG. 8.—The relation between frequency of ingestion of chilomonads by *A. proteus* and osmotic pressure. Ordinates, number of chilomonads ingested; abscissae, molar concentration of solutions; ●, number of chilomonads ingested in 15 minutes; ◉, 30 minutes; ◑, 60 minutes; ◓, 120 minutes. Each point on the curves represents the average for 10 individuals.

The fact that the frequency of ingestion was higher in Hahnert solution plus lactose than in Hahnert solution indicates that ingestion is correlated with osmotic concentration. The fact that it was zero in lactose solutions and higher in Chalkley solution than in Hahnert solution plus lactose (0.03 M), which was osmotically equal to Chalkley solution, indicates that the effect of salts on ingestion is not entirely due to their osmotic action. The marked difference in the frequency of ingestion in Chalkley and Hahnert solutions is doubtless due to the fact that the former contains NaCl and the latter does not.

DISCUSSION

Mast made a thorough study of the effect of salts, nonelectrolytes, and hydrogen ion concentration on attachment of *A. proteus* to the substratum. He maintains that nonelectrolytes have no effect on attachment, but that all salts greatly facilitate it and that this is not closely correlated with their concentration or the hydrogen ion concentration of the solutions. He says (1929, p. 375):

> All the salts tested (17 in number) greatly decrease the time required for attachment and increase the firmness of attachment. All have the same effect but not in the same concentration
>
> The effect of NaCl and KCl increases very gradually from N/20 to a maximum at about N/100, after which it decreases very gradually until about N/1000 is reached and then very rapidly. The effect of $CaCl_2$ increases rapidly from N/20 to about N/60 then slowly to a maximum at about N/200; after which it decreases very slowly until about N/40,000 is reached and then very rapidly. There is very little difference in the effect on attachment of NaCl and KCl in concentrations ranging from N/40 to N/800 and of $CaCl_2$ in concentrations ranging from N/80 to N/40,000. The effect of $CaCl_2$ is considerably less than that of NaCl and KCl in the stronger and much greater in the weaker solutions tested. All three salts, however, still have a definite effect in the weakest solution tested, N/100,000.
>
> The optimum hydrogen ion concentration for attachment in balanced solution appears to be between pH 6.6 and 7; but there is remarkably little difference over a very wide range, i.e. from pH 4.6 to 8.2.

He found no indication whatever of a median minimum at neutrality.

By comparing these results with those presented in the preceding pages, it is at once evident that, while the effect of nonelectrolytes on attachment seems to be the same as it is on ingestion, the effects of salts and hydrogen ions on ingestion differ greatly from their effects on attachment, and that this is particularly striking in reference to a hydrogen ion concentration of pH 7, in which ingestion was found to be maximum and attachment minimum.

This strongly indicates that, while ingestion is doubtless dependent upon attachment, in accord with the views of Mast and Hahnert (1935), these two phenomena are not closely correlated.

We have demonstrated that the relations between temperature, properties and concentration of salts, and hydrogen ion concentration and frequency of ingestion of food by *Amoeba* are essentially the same as the relation between these environmental factors and the rate of locomotion. This indicates that the factors which control ingestion are fundamentally the same as those which control locomotion.

Pitts and Mast (1934) considered in some detail the mode of action of environmental factors in controlling locomotion. They accounted for some phases of the phenomena involved; but they could give no consistent explanation of the relation between hydro-

gen ion concentration and rate of locomotion in balanced salt solution, especially the extraordinary reduction in rate at neutrality.

We have no suggestions to offer concerning the mode of action of environmental factors in controlling ingestion except that it probably is essentially the same as it is in reference to locomotion, and we have nothing of value to add to the discussion of Pitts and Mast concerning this.

The results presented demonstrate clearly that in optimum concentration sodium is much more favorable for ingestion than potassium, calcium, or magnesium. We have no suggestions concerning the causes of this.

SUMMARY

1. A quantitative method was devised and used in studying the frequency of ingestion of food by amoebae.

2. Frequency of ingestion varies inversely with the amount of food in the amoebae, but there can be large variations in the abundance of food in the surrounding medium without appreciably affecting frequency of ingestion.

3. As the temperature increases, frequency of ingestion increases from zero at 0° C to a maximum at 25°, and then decreases to zero at about 40°. There is some evidence of a median minimum at 30°.

4. As the hydrogen ion concentration decreases in solutions in which sodium is the only metallic cation, frequency of ingestion increases from zero at pH 4.4 to a maximum at approximately pH 6.0, then decreases to zero at pH 7.6 to 8.3. There is strong indication of a median minimum between pH 6.5 and 7.0. The results indicate that in solutions in which the concentration of sodium is 0.005 N, frequency of ingestion is closely correlated with the hydrogen ion concentration, but not in solutions in which the concentration of sodium is 0.002 N.

5. The relation between hydrogen ion concentration and frequency of ingestion in solutions in which potassium is the only metallic cation is similar to that in solutions in which sodium is the only metallic cation, but the range over which ingestion occurs is much shorter and the frequency of ingestion is much lower.

6. In solutions in which calcium is the only metallic cation, frequency of ingestion increases from about 0.9 chilomonads per minute at pH 4.7 to a maximum of 1.8 chilomonads per minute at approximately pH 6.5, then decreases very slowly until pH 8.0 is reached, and then drops rapidly to zero.

7. In solutions in which sodium and calcium are the only metallic cations, frequency of ingestion increases very rapidly from zero at pH 4.5, to a maximum at pH 6.2 then decreases to a marked median minimum at pH 7.0, then increases to a secondary maximum approximately at pH 7.7, then decreases nearly to zero at pH 8.4. The median minimum and the secondary maximum in the alkaline region of the range are much more pronounced in solutions containing Na 0.005 N and Ca 0.003 M than in solutions containing Na 0.005 N and Ca 0.001 M.

8. In solutions of NaCl, as the concentration increased, frequency of ingestion increased very rapidly from about 0.49 chilomonads per minute at 0.0003 M, to a maximum of 1.54 chilomonads per minute at 0.0008 M, and then decreased slowly to zero at 0.03 M.

9. The relation between concentration and frequency of ingestion in solutions of KCl is essentially the same as in solutions of NaCl; but the frequency of ingestion is much

lower, and the range over which ingestion occurs is only about one-third as long, the maximum concentration being 0.03 M in the one and only 0.01 M in the other.

10. In solutions of $MgCl_2$, as the concentration increases, the frequency of ingestion increases rapidly from 0.59 chilomonads per minute at 0.0001 M, to a maximum of 0.83 chilomonads at 0.0005 M, and then decreases gradually to zero at 0.005 M.

11. The relation between concentration and frequency of ingestion in solutions of $CaCl_2$ is essentially the same as in solutions of $MgCl_2$ except that frequency in the lowest concentration tested is considerably higher.

12. The range of concentration over which ingestion occurs in solutions of $MgCl_2$ or $CaCl_2$ is only about one-sixth as long as that over which it occurs in solutions of NaCl. The cause of this difference is not clear.

13. The relation between temperature, salts, and hydrogen ion concentration and the frequency of ingestion of food is essentially the same as the relation between these environmental factors and rate of locomotion. Frequency of ingestion and rate of locomotion are therefore closely correlated.

14. Frequency of ingestion of chilomonads by *A. proteus* and *A. dubia* is zero in solutions of lactose in all concentrations, but is it higher in Hahnert solution plus lactose than in Hahnert solution without lactose. This indicates that osmosis is involved in feeding.

15. The frequency of ingestion is much higher in Chalkley solution than in Hahnert solution plus lactose 0.003 M (osmotically equal to Chalkley solution). This is doubtless due to the fact that the former contains NaCl and the latter does not.

16. The frequency of ingestion is much higher in *A. dubia* than in *A. proteus*. The maximum number ingested by an individual of the former was 100 in 15 minutes, 195 in 30 minutes, 366 in 60 minutes, and 405 in 120 minutes; and by one of the latter, only 48 in 15 minutes, 95 in 30 minutes, 130 in 60 minutes, and 250 in 120 minutes.

LITERATURE CITED

BLASDALE, W. C. 1931. The fundamentals of quantitative analysis. New York. Pp. 403.

CLARK, W. 1928. The determination of hydrogen ions. Baltimore. Pp. 717.

FARBER, J. E., and YOUNGBURG, G. 1932. Determination of phosphates in waters. Ind. and Eng. Chem., **4**:107–11.

FENN, W. O. 1921. The phagocytosis of solid particles. II. Carbon. Jour. of Gen. Physiol., **3**:465–82.

HAHNERT, W. F. 1932. Studies on the chemical needs of *Amoeba proteus:* A culture method. Biol. Bull., **62**:205–11.

HARGITT, G. T., and FRAY, W. W. 1917. The growth of *Paramecia* in pure cultures of bacteria. Jour. Exp. Zoöl., **22**: 421–25.

HEILBRUNN, L. V., and DAUGHERTY, KATHRYN. 1932. The action of sodium, potassium, calcium and magnesium ions on the plasmagel of *Amoeba proteus*. Physiol. Zoöl., **5**:254–74.

HOPKINS, D. L. 1928. The effect of certain physical and chemical factors in locomotion and other life processes in *Amoeba proteus*. Jour. Morph. and Physiol., **45**: 97–119.

MAST, S. O. 1926. Structure, movement, locomotion and stimulation in *Amoeba*. *Ibid.*, **61**:347–425.

———. 1929. Mechanics of locomotion in *Amoeba proteus* with special reference to the factors involved in attachment to the substratum. Protoplasma, **8**:344–77.

MAST, S. O., and HAHNERT, W. F. 1935. Feeding, digestion, and starvation in *Amoeba proteus*. Physiol. Zoöl., **8**:252–72.

MAST, S. O., and PROSSER, C. L. 1932. The effect of temperature, salts, hydrogen ion concentration on rupture of the plasmagel sheet, rate of locomotion, and gel/sol ration in *Amoeba proteus*. Jour. Cell. and Comp. Physiol., **1**:333–54.

METALNIKOW, S. 1912. Contributions à l'étude de la digestion intracellulaire chez les protozoaries. Archiv. de zool. exp., **9**:373–498.

PHILLIPS, RUTH L. 1922. The growth of *Paramecium* in infusions of known bacterial content. Jour. Exp. Zoöl., **36**:135–83.

PITTS, R. F. 1932. Constant temperature apparatus adapted for use on the microscopic stage. Science, N.S., **76**:626–27.

PITTS, R. F., and MAST, S. O. 1934. The relation between inorganic salt concentration, hydrogen ion concentration, and physiological processes in *Amoeba proteus*. II. Rate of locomotion, gel/sol ratio, and hydrogen ion concentration in solutions of single salts. Jour. Cell. and Comp. Physiol., **4**:237–56.

———. 1934. The relation between inorganic salt concentration, hydrogen ion concentration, and physiological processes in *Amoeba proteus*. III. The interaction between salts (antagonism) in relation to hydrogen ion concentration and salt concentration. *Ibid.*, pp. 435–55.

SCHAEFFER, A. A. 1910. Selection of food in *Stentor coeruleus* Ehr. Jour. Exp. Zoöl., **8**:75–132.

———: 1916. On the feeding habits of *Amoeba*. *Ibid.*, **20**:529–48.

SCHWITALLA, A. M. 1925. The rate of locomotion in *Amoeba* at different temperatures. Jour. Morph. and Physiol., **41**:45–58.

THE EFFECT OF CHEMICALS AND TEMPERATURE ON REVERSAL IN CILIARY ACTION IN PARAMECIUM[1]

(One figure)

JOSEPH F. OLIPHANT
Stanford University

REVERSAL in direction of the effective stroke of the cilia is very common in the protozoa and occurs frequently in the metazoa, but only certain phases of it have been adequately investigated, and few attempts have been made to ascertain the physiological and environmental factors involved in the phenomenon.

Jennings (1899*a*) found that a variety of stimuli (chemical, mechanical, electrical, and thermal) cause *Paramecium* momentarily to reverse its direction of locomotion. He further noted (1899, p. 34) that in paramecia put into a 1 per cent solution of KI the action of the cilia reverses for 6–8 minutes.

Parker (1896, 1905*a*, 1905*b*) found the action of the cilia on the oral disk of *Metridium marginatum* reverses in response to dilute crab-meat juice, a sea-water extract of rubber, 2, 3, 4, and 5 per cent solutions of KCl in sea water, a 5/8 M solution of NaCl containing M/15 or M/30 creatin, to peptone or deutero-albumine, and asparaginic acid. He did not obtain reversal using NaCl alone, LiCl, NHCl, $NaNO_3$, a number of sugars, urea, creatinine, or chemically inert substances such as bits of filter paper or sand. Before he had worked with organic compounds other than crab-meat juice, he said (1905*a*, p. 13): "Reversal is due to potassium ions or to some organic combination containing potassium ions." After having observed the effects of various organic compounds, he modified these statements and said (1905*b*, p. 5): "The reversal . . . . is due cheifly to molecular action, though potassium ions if sufficiently concentrated will cause reversal."

Merton (1923) obtained reversal of ciliary action in *Stentor coeruleus* in solutions containing Na and K ions but did not obtain reversal in solutions of $CaCl_2$, $MgSO_4$, and $MgCl_2$. He suggested that the reversal is due to the Na and K ions and concluded (p. 13):

> Wenn der Zusatz von K oder Na Ionien eine Umkehr der Flimmerbewegung hervorruft, so besteht die Möglichkeit, das unter naturlichen Bedingungen diese Ionien selbst in dem Stentor wirksam sind. Es kann aber auch und das ist wahrschleinlicher ein unbekannter Stuff diese Reaktion hervorrufen und nur im Versuch durch diese Kationen ersetzt werden.

Merton (1935) found in further studies with *Stentor* that a number of salts of K, Na, Rb, Ce, and NH_4 induce reversal in the action of the cilia. Reversal, however, was not obtained with calcium salts. He says that reversal results from some specific action of the alkali metals, though he does not say what the specific action is.

Mast and Nadler (1926) investigated the relation between fifty-six different chemicals in the environment and reversal in ciliary action in *Paramecium caudatum*. By observing the direction of locomotion, they ascertained qualitatively the effect of the chemicals

[1] Contribution from the Zoölogical Laboratory, Johns Hopkins University. The author wishes to acknowledge his indebtedness to Professor S. O. Mast for valuable suggestions concerning the experiments and for valuable assistance in the preparation of the manuscript.

in inducing reversal. They found that all the univalent salts and hydrates tested induce reversal except $(NH_4)_2SO_4$ and $(NH_4)C_2H_3O_2$ but none of the bivalent and trivalent cation salts and hydrates except $CaHPO_4$, $MgHPO_4$ and $Ba(OH)_2$. Polyvalent cation salts were found to neutralize the effect of monovalent cation salts. They conclude (p. 117): "Ciliary reversal is associated with differential absorption of cations and subsequent changes in electrical potential, but there are also other factors involved."

Eisenberg-Hamburg (1932) reported a *Rückwärtsbewegungreaktion* when *Paramecium* is introduced into solutions of salts of Sr, Rb, and Ce. She maintains that this reaction consists of a series of backward and forward movements extending over considerable periods of time and that the reversed phase is in each instance of very short duration. The reaction was also obtained, though not in such a characteristic manner, in Ba salts but was not obtained in Mg, Ca, Cd, Na, K, and Fe salts. Similar results were obtained with *Colpidium*, *Stentor*, and *Spirostomum*. She concludes (p. 123): "Die Rückwärtsbewegungreaktion bildet somit eine Reflexbewegung des normalen Infusors, hervorgerufen durch die Anwesenheit des Sr Kations [and by inference other cations] dessen chemisch-physikalischen Wirkung aller Wahrscheinlichkeit nach die Kollöide der Zelle angreift."

It is the object of the present investigation to ascertain more precisely the relation between monovalent cation salts in the environment and reversal in the effective beat of cilia with the view of throwing light on the physiological factors involved in ciliary reversal.

MATERIALS AND METHODS

For this investigation *Paramecium multimicronucleatum* was chosen owing to its size and the readiness with which it lends itself to culture and experimentation. The specimens used were all obtained from a clone cultured as follows: 7 gr. of timothy hay in a liter of Chattolanee spring water were heated and kept at the boiling-point for 3 minutes; then 200 cc. of this infusion were poured into each of five Erlenmeyer flasks, which were then stoppered with cotton plugs and left 12 hours, after which 5 cc. of old infusion containing paramecia were added to each flask. New cultures were prepared each week, and no culture was used for experimentation until after it had stood for a week. By this time the hydrogen-ion concentration of the culture was pH 6.9 ± 0.1. The temperature of the room in which the cultures were grown varied between 21° and 23° C., inclusive. By this method animals in nearly the same physiological condition were obtained for the experiments.

The chemicals used were Kahlbaum's "pro-analyse" and Merck's blue-label Reagent. The chemical solutions were made with Chattolanee spring water. This spring water was found to be more favorable than any other solution used. Distilled water has been used by other investigators, but it is not as satisfactory as spring water, for, when *Paramecium* is transferred from a culture medium to distilled water, action of the cilia is reversed. It is therefore necessary to acclimatize specimens to distilled water before making tests of effects of chemicals. This acclimatization must be done by gradual dilution over a period of 12 hours or more and even then there are not infrequently produced ruptures of the ectosarc by the entrance of water.

Merton (1935) used tap water in his studies on *Stentor*. In preliminary experiments of the present work it was found that various tap waters and spring waters can be used but that few of them are satisfactory because of great seasonal variation in content.

Chattolanee spring water is obtained from covered springs, and chemical analyses show that its variation in total salt content throughout the year is only about 1 milligram per cent.[2] It tends to act as a natural buffer, and it has no appreciable effect on *Paramecium*. Thoroughly washed specimens live in it for as long as 10 days.

In making the tests, paramecia were first thoroughly washed in spring water, transferred to pure spring water, and left for an hour before being used for experimentation. To ascertain the effects of chemicals on ciliary reversal, 2.5 cc. of solution to be tested were put into a Columbia pyrex glass culture dish on the stage of a dissecting binocular microscope. One of the washed paramecia was taken up in a capillary pipette, care being exercised to take as little water as possible, and dropped into the test solution. The specimen was then observed continuously until reversed action of the cilia ceased and the duration of reversal measured with a stop watch. This was repeated with other specimens as often as was desired.

Numerous experiments by various investigators demonstrate that reversal in the action of the cilia in *Paramecium* is sometimes confined to localized regions of the body. In all the experiments presented here ciliary reversal was only recorded as having occurred if the specimens actually swam backward for a time measurable with a stop watch.

The method described above was slightly modified in some experiments as indicated in the following pages.

RELATION BETWEEN CONCENTRATION OF MONOVALENT CATION SALTS AND REVERSAL IN CILIARY ACTION

The time of backward swimming was ascertained for ten specimens in each of a series of concentrations of the chlorides, iodides, and bromides of sodium, potassium, lithium, and ammonium. The hydrogen-ion concentration of the chemical solutions used was pH 7.4 $\pm$ 0.1, that of the culture medium from which the organisms were taken pH 6.9 $\pm$ 0.1, and that of the wash solution to which the specimens were adapted pH 7.4. The temperature varied between 21° and 23°C.

In molar solutions in NH_4Br, NH_4Cl, and NH_4I the specimens died immediately without reversal, while in all the other salts they died in 1–2 seconds and swam backward continuously. In M/5 and M/10 NH_4I death was immediate, and there was no reversal. In M/5 and M/10 LiI and LiBr death occurred in about 10 seconds, and the paramecia swam backward continuously. In M/5 concentrations of the other salts death occurred in 2–8 minutes, and there was an initial period of reversed ciliary action which was followed by momentary recovery and normal forward movement after which there were repeated reversals and recoveries until death. In M/5 NaBr, for example, the animals swam backward 9.45 $\pm$ seconds then forward 3–5 seconds then backward 0.5–1.0 seconds then forward 1–3 seconds and then continued to reverse at this frequency about 2–3.5 minutes, i.e., until they died, which usually, but not always, occurred in a reversed phase of about 30 seconds' duration. This type of behavior occurred in the other sodium salts and in M/5 and M/10 LiCl. In higher concentrations the animals, immediately before they died, revolved in a plane about the posterior end without rotating on the longitudinal axis. In other concentrations of all salts tested the initial resumption of forward movement was seldom followed by subsequent reversals. Re-

[2] Composition of Chattolanee spring water in grams of salt per liter: NaCl, 0.0034; Na_2CO_3; 0.00013; K_2SO_4, 0.0017; $CaSO_4$, 0.00007; $MgCO_3$, 0.020; $CaCO_3$, 0.052; silica, 0.0092; oxides of Fe and Al, 0.00066.

covery from a reversed condition in these concentrations was always preceded by a type of behavior in which the anterior end of the animal moved in a great circle while the posterior end moved in a small circle. Forward movement in recovered specimens was a little slower than in animals not subjected to the action of a chemical.

In M/400 of all the salts tested except those of ammonium there was a pronounced motor response accompanied by momentary reversal of such short duration that it could not be measured with a stop watch. In M/1,000 of the various salts except those of ammonium paramecia gave a slight motor response, but in no instance was there anything that could be considered true ciliary reversal. Similar results were obtained in ammonium salts M/50–M/150.

The results of one complete series of experiments are summarized in Table 1 and Figure 1. The table and figure show that in solutions of chlorides, iodides, and bromides of sodium, potassium, lithium, and ammonium duration of reversed ciliary action varies directly with concentration of the salt and that the order of effectiveness of the monovalent cations in inducing ciliary reversal is $K > Li > Na > NH_4$. A typical series of readings from which the average duration of reversal was computed, the one obtained in M/10 NaCl, the first salt in the table, follows: 11.1, 10.8, 11.2, 12.5, 11.9, 12.0, 10.5, 10.1, 8.4, and 9.6 seconds. The variation in individual readings was somewhat greater in bromides and iodides of potassium and lithium. This is shown typically by the results obtained with M/10 KBr which are 28.2, 32.5, 28.8, 27.6, 27.9, 27.1, 44.3, 25.6, 37.5, and 36.1 seconds. These series indicate considerable variation; however, in columns 4, 5, and 6 of the table it is revealed that the standard deviation is small in comparison with the average time of reversed ciliary action and that the probable error is very small, rarely exceeding 6 per cent. The standard deviation and percentage of probable error are greatest in the iodides, less in the bromides, and least in the chlorides. The reason for this variation is not clear.

All the experiments summarized in Table 1 and Figure 1 were repeated twice, and the results obtained are qualitatively and quantitatively in agreement with those presented.

The conclusions of these experiments are in accord with the conclusion of Eisenberg-Hamburg (1932) that the *Rückwärtsbewegungreaktion* is greater, the greater the concentration of the salt inducing it, and they are supported by the results obtained by Merton (1935) on *Stentor*, which indicate that duration of reversal varies directly with concentration; but they are not in accord with the results obtained by Mast and Nadler on observations on *P. caudatum*. They say (1926, p. 116):

> The duration of reversed action varies with the kind of salt and with the concentration. In KCl as the concentration decreases, the duration of this action increases from zero at M/10 to a maximum of about 40 seconds at M/750, then decreases to zero at M/2000. In NaCl it increases from zero at M to a maximum of about 14 seconds at M/500, then decreases to zero at M/2200. In NH_4Cl it increases from zero at M/50 to a maximum of about 10 seconds at M/1000, then decreases to zero at M/2200. In LiCl it increases very rapidly from zero at M/5 to a maximum of about 55 seconds at M/10, then decreases slowly to zero at M/2000.

The results presented above are obviously not in accord with these claims, for they show that in solutions of KCl, LiCl, and NH_4Cl duration of reversed ciliary action varied directly with concentration and that no reversal in ciliary action was obtained in concentrations between M/500 and M/750, the optimum for reversal found by Mast and Nadler.

TABLE 1

RELATION BETWEEN DURATION OF REVERSED CILIARY ACTION IN *Paramecium* AND CONCENTRATION OF MONOVALENT CATION SALT INDUCING REVERSAL

"Average," time of reversal for ten specimens; S.D., standard deviation; P.E., probable error; percentage P.E., percentage probable error of the average duration of reversal.

Salt	Concentration	Average	S.D.	P.E.	Percentage P.E.
NaCl	M/10	10.81	1.81	0.25	2.3
	M/20	7.49	0.80	0.16	2.1
	M/50	5.73	0.87	0.18	3.1
	M/100	6.04	1.40	0.29	4.7
	M/200	4.26	0.77	0.16	3.7
KCl	M/10	43.82	3.03	0.64	1.4
	M/20	30.05	3.59	0.74	2.4
	M/50	14.22	3.56	0.74	5.2
	M/100	3.92	0.95	0.20	5.1
	M/200	1.00	Not calculated		.....
LiCl	M/10	13.22	1.88	0.40	3.1
	M/20	9.00	1.24	0.26	2.8
	M/50	7.52	1.80	0.38	5.0
	M/100	5.28	0.86	0.18	3.4
	M/200	3.52	0.55	0.11	3.1
NH_4Cl	M/5	21.20	2.25	0.47	2.2
	M/10	8.70	1.78	0.37	4.2
	M/20	2.10	0.38	0.80	3.8
	M/50	1.00	Not calculated		.....
	M/100	?	.....	.....	.....
NaBr	M/10	8.12	1.73	0.33	4.0
	M/20	5.63	1.24	0.21	3.7
	M/50	3.74	1.11	0.25	6.6
	M/100	1.00	Not calculated		.....
	M/200	?	.....	.....	.....
KBr	M/10	31.56	5.92	1.26	5.1
	M/20	29.78	8.62	1.80	6.0
	M/50	9.25	2.27	0.48	5.1
	M/100	3.14	0.78	0.16	3.9
	M/200	1.00	Not calculated		.....
LiBr	M/10	8.59	1.53	0.26	3.0
	M/20	13.25	5.15	1.63	12.1
	M/50	5.71	0.20	0.13	2.1
	M/100	3.89	1.05	0.22	5.6
	M/200	1.00	Not calculated		.....
NH_4Br	M/5	10.62	2.81	0.59	5.5
	M/10	3.97	1.65	0.34	8.3
	M/20	1.00	Not calculated		.....
	M/50	?	.....	.....	.....
NaI	M/10	7.53	2.78	0.59	7.9
	M/20	4.74	0.70	0.14	2.9
	M/50	3.47	1.20	0.24	6.8
	M/100	2.95	1.02	0.21	7.1
	M/200	1.00	Not calculated		.....

TABLE 1—*Continued*

Salt	Concentration	Average	S.D.	P.E.	Percentage P.E.
KI	M/10	53.36	14.37	3.06	5.7
	M/20	49.92	15.26	3.24	6.4
	M/50	12.57	3.46	0.73	5.7
	M/100	4.16	1.02	0.21	5.0
	M/200	1.00	Not calculated		
LiI	M/10	14.09	3.95	0.84	5.2
	M/20	13.95	4.25	0.90	6.4
	M/50	6.02	1.95	0.41	6.8
	M/100	4.43	1.70	0.36	8.1
	M/200	1.00	Not calculated		
NH_4I	M/10	Death without reversal			
	M/20	5.35	1.35	0.24	4.4
	M/50	8.22	1.03	0.21	3.2
	M/100	6.49	0.97	0.20	3.0
	M/200	?			

The differences in results of the two investigations may have been due to differences in physiological states of the organisms traceable to conditions in the infusions in which they were grown and to adaptation to different wash solutions. Mast and Nadler used *P. caudatum* adapted to distilled water. The hydrogen-ion concentrations were not controlled, and the temperatures are not given. The differences are not due to the fact that different species were used, for in tests of *P. caudatum* in KI, KCl, and KBr under conditions of the present investigation results were obtained which are essentially the same as those obtained with *P. multimicronucleatum*.

THE ROLE OF ANIONS IN CILIARY REVERSAL

The possible role of the anions used in the present experiments was considered. When the data presented in Table 1 are compared, it is found that the difference in effect produced by like concentrations of chloride, bromide, and iodide having the same cation is in all instances slight, and it is impossible to recognize a consistent order in which these anions affect ciliary reversal.

To investigate further the possible role of anions, the effect of some bivalent cation salts was tested. Calcium and magnesium were used because they are closely related chemically to the alkali metals, whose salts induce reversal, and they are among the more abundant bivalent cations found in and having pronounced effects upon various types of protoplasm. Chlorides, bromides, and iodides of calcium and magnesium were tested from M/1 to M/20,000. In concentrations from those not immediately lethal about M/250 up to M/2,500 of each salt various types of motor reactions were observed, but in no instance was there anything that could be considered true ciliary reversal. These experiments indicate that calcium and magnesium ions do not induce reversal in ciliary action in *Paramecium* and that chlorine, bromine, and iodine ions do not induce reversal and that their action in reversal is quantitative. No other anions were investigated. However, Merton (1935) in experiments on *Stentor* presents evidence which indicates that some salts of potassium are more effective in inducing ciliary reversal than others. He lists the anions used above in the order Cl > I > Br.

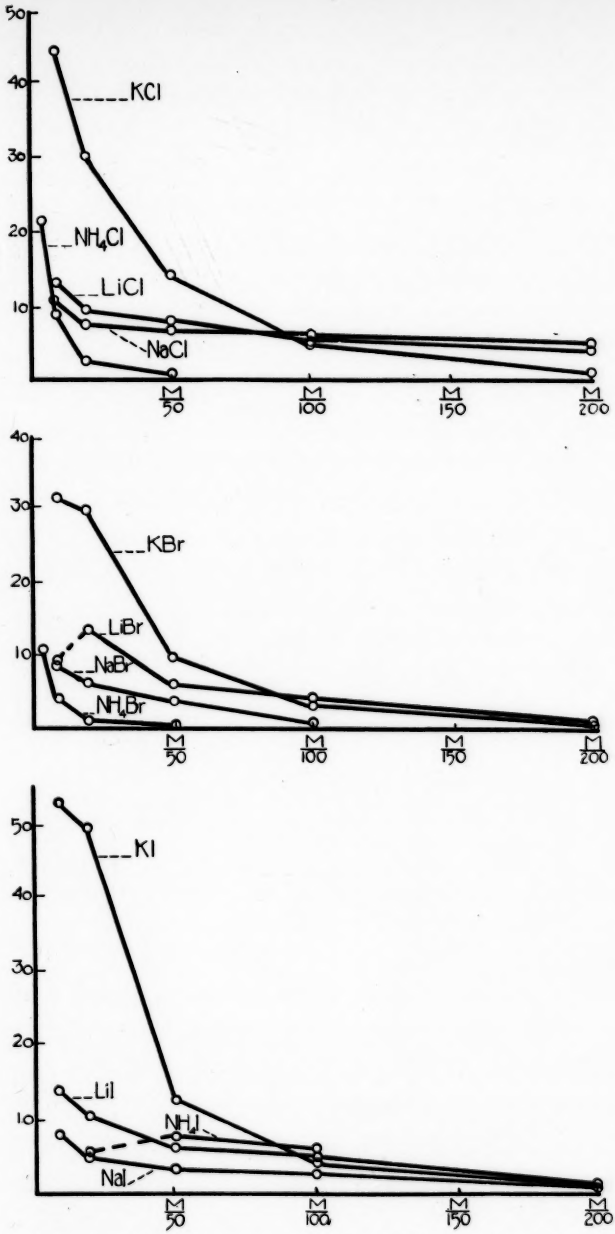


FIG. 1.—Graphs showing the relation between duration of reversed ciliary action in *Paramecium* and concentration of various monovalent cation salts inducing reversal. Ordinates, time of reversed ciliary action in seconds; abcissae, concentration of salt.

THE EFFECT OF ADAPTATION ON CILIARY REVERSAL INDUCED BY SOLUTIONS OF MONOVALENT CATION SALTS

The question whether ciliary reversal in *Paramecium* is dependent on the absolute concentration of monovalent cation salt in the environment or upon rate of change (increase) in concentration has a direct bearing on adaptation. It was investigated as follows: Numerous paramecia were thoroughly washed in spring water; then 6 cc. of solution containing washed paramecia were added to 6 cc. of a monovalent cation salt solution M/20 in a 25 cc. stender dish. The dish was then covered and left for 18 hours. These paramecia and others which were washed but had not been in the salt solution were then tested for reversal in ciliary action in a number of concentrations of the salt to which the experimental animals had been exposed. This experiment was repeated with the chlorides, iodides, and bromides of sodium, lithium, and potassium. Care was exercised to adapt approximately the same number of organisms in each experiment.

It was found that *Paramecium* becomes adapted (no longer swims backward) to M/40 solutions of all salts tested in less than 18 hours, otherwise the adapted paramecia behaved essentially like those which were not adapted; however, the behavior was not the same in all salts. The paramecia adapted to lithium and potassium salts moved slightly slower and were darker and much more vacuolated than the controls. A microscopic examination revealed an apparent increase in protoplasmic consistency, and there was evidence of slight plasmolysis. Specimens adapted to sodium salts behaved more nearly like the controls; however, they were darker in appearance and more vacuolated. No difference could be detected between paramecia adapted to chlorides, iodides, and bromides having the same cation. After paramecia had remained two days and longer in lithium and potassium salts, movement was accompanied by active changes in shape, was very slow in comparison with controls, and reversals in the direction of rotation on the longitudinal axis increased. The paramecia in lithium salts died first, then those in potassium salts, then those in sodium salts. The order in which the cations produce death therefore seems to be in no way correlated with the order in which they induce reversal in ciliary action. Observations were not made at sufficiently frequent intervals to ascertain the order of the anions.

The rate of reversed beat of the cilia was observed to differ in the different salts, but no actual measurements were made. However, it could be seen that it was greatest in lithium, less in potassium, and least in sodium salts. This is not in accord with the order of effectiveness of these cations in inducing ciliary reversal, but it is in accord with the order in which they produce death.

Some of the results of the experiments are presented in Table 2. This table shows that in paramecia adapted to a M/40 solution of a given monovalent cation salt ciliary reversal does not occur in lower concentrations (M/50 and M/100) but does occur in higher concentrations (M/20, M/10, and M/5). It shows further that in paramecia not adapted to a given monovalent cation salt ciliary reversal occurs in all concentrations from M/100 to M/5 and that the duration of reversed ciliary action in a given salt concentration is greater in nonadapted than in adapted specimens. These facts show that it is the rate of change (increase) and not absolute concentration of monovalent cation salt in the environment that induces reversal in the action of the cilia.

THE EFFECT OF TEMPERATURE ON CILIARY REVERSAL

Strasburger (1878) found that swarm spores which are photopositive in a given light intensity at 16°–18° C. become negative when the temperature is decreased to 4° C.

and more strongly positive when it is increased to 35° C. Massart (1891) obtained similar results in observations on *Chromulina* and Mast (1911) in observations on *Euglena, Chlamydomonas, Trachelomonas, Chlorogonium,* and *Volvox;* but no observations have been made on the effect of change in temperature on reversal in ciliary action.

In making this investigation, the temperature was controlled within 0.5° C. by means of temperature chambers on the stage of a low-power binocular microscope (Pitts, 1932). In making the tests, paramecia were thoroughly washed and then left 1½ hours in spring water at a given temperature in a temperature chamber on the stage of a binocular microscope. One individual was then taken up in a capillary pipette and put into 5 cc. of solution to be tested (in another temperature chamber on the stage of another micro-

TABLE 2

EFFECT OF ADAPTATION TO SALT ON DURATION OF REVERSED CILIARY ACTION IN *Paramecium.*

Each number in the table is the average of the duration in seconds of reversal for ten specimens *Ins.* indicates reversal too short in duration to be measured with a stop watch.

Concentration	Average Duration in Seconds of Reversed Ciliary Action								
	NaI	NaBr	NaCl	LiI	LiBr	LiCl	KI	KBr	KCl
	Paramecia adapted to M/40 Salt Solutions								
M/100	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
M/50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
M/20	1.24	0.75	1.29	2.10	4.62	4.02	24.15	5.49	1.39
M/10	2.47	2.67	2.51	5.07	8.33	7.00	41.62	13.91	6.60
M/5	6.41	3.82	7.29	5.10	23.79	17.67	55.17	16.01	9.77
	Paramecia Not Adapted								
M/100	Ins.	Ins.	2.07	2.59	3.33	4.13	3.21	2.19	2.11
M/50	2.22	2.87	4.62	5.46	6.18	6.33	26.14	8.16	8.56
M/20	5.10	4.15	6.18	12.17	11.23	8.45	43.52	28.14	24.34
M/10	8.27	9.11	8.76	15.24	13.82	13.27	51.14	37.64	32.67
M/5	11.31	11.27	11.39	12.00	18.10	7.53	73.46	45.58	41.21

scope) at any temperature desired, then the behavior of the specimen was observed continuously until the end of the experiment. If there was ciliary reversal, the duration was measured with a stop watch. This procedure was repeated as often as desired. Observations were made on ten specimens at each temperature tested.

Paramecia were left in spring water at 23° C. for 1½ hours, then transferred to spring water at 7°, 15°, 23°, 30°, 40°, and 50° C. The following was observed: at 7°–30° there were violent motor reactions but no continuous backward swimming. At 40° C. there was ciliary reversal which continued 1.5 seconds. At 50° the paramecia died immediately. In paramecia adapted to 15° and 30°, respectively, similar results were obtained. The paramecia did not become adapted to 7°, 35°, and 40° C.

These experiments indicate that reversal in ciliary action occurs only after transfer to temperatures almost immediately lethal and then continues only a very short time.

Paramecia were adapted for 1½ hours to spring water at 23° C. then put into 5 cc. of various chemical solutions at 7°, 15°, 23°, 30°, 40°, and 50° C. The results obtained are presented in Table 3.

Table 3 shows that duration of reversed ciliary action in M/20 and M/50 solutions of all the salts tested varies inversely with temperature and that in a given concentration of chloride, bromide, or iodide of potassium, sodium, and lithium at a given temperature the order of effectiveness of the monovalent cations in inducing reversal is K > Li > Na.

TABLE 3

RELATION BETWEEN DURATION OF REVERSED CILIARY ACTION IN *Paramecium* IN M/20 AND M/50 SOLUTIONS OF SALTS

Each number in the table represents the average duration of reversal in seconds in ten specimens. Death was immediate without reversal.

Salt	Concentration	Temperature					
		7° C.	15° C.	23° C.	30° C.	40° C.	50° C.
KCl	M/20	68.63	42.09	28.20	20.36	16.81	Death
KCl	M/50	48.12	20.86	11.58	6.25	3.80	"
LiCl	M/20	77.31	32.80	8.94	4.89	3.71	"
LiCl	M/50	65.91	24.14	6.57	3.69	2.23	"
NaCl	M/20	23.29	11.79	5.60	2.80	1.61	"
NaCl	M/50	18.94	9.53	4.22	1.86	1.24	"
KBr	M/20	83.89	52.44	27.39	19.53	8.29	"
KBr	M/50	39.27	21.63	12.15	4.81	3.20	"
LiBr	M/20	78.33	29.56	11.72	5.32	4.18	"
LiBr	M/50	51.12	19.27	6.52	5.15	4.78	"
NaBr	M/20	22.89	11.91	5.37	3.99	1.76	"
NaBr	M/50	17.62	9.21	4.47	2.26	2.15	"
KI	M/20	97.30	44.21	30.76	12.92	8.22	"
KI	M/50	43.97	16.81	11.21	5.38	3.36	"
LiI	M/20	87.49	31.20	13.18	5.26	3.67	"
LiI	M/50	44.34	16.51	7.16	2.94	2.72	"
NaI	M/20	35.62	15.41	7.06	4.39	2.69	"
NaI	M/50	26.43	9.27	4.21	3.53	2.47	"

Comparison of relation between duration of reversal in a given concentration of chlorides, bromides, and iodides having the same cation and temperature indicates that, if these anions affect ciliary reversal, there is no constant or significant order in which they affect it. The similarity of effects produced by salts of the same cation but different anions is particularly striking in the salts of lithium given in Table 3.

There were marked differences in rate at which paramecia swam backward at different temperatures. The rate was very slow at 7° C., somewhat more rapid at 15° C., rapid at 23°, very rapid at 30°, and extremely rapid at 40° C. Actual measurements were not made, but careful observation indicates that it is probably directly proportional to the temperature.

Paramecia exposed to the lower temperatures became darker in appearance and more vacuolated, and the granular nature of the endoplasm was more pronounced. Death in high temperatures was accompanied by increased vacuolation change in shape and coagulation of the cytoplasm.

Similar results were obtained with paramecia adapted to 15° and 30° C.

DISCUSSION

It is demonstrated above that *Paramecium* reacts to increase in monovalent cation salt concentration in the environment by reversal in action of the cilia but does not react to increase in bivalent cation salt concentration. This difference in response must be due to some difference in the effect of monovalent and bivalent cations on the protoplasm.

Heilbrunn (1923) found chlorides of calcium and magnesium cause a decrease, while chlorides of potassium, sodium, and ammonium cause an increase in protoplasmic viscosity of eggs of *Arbacia*. He (1923) obtained similar results with *Stentor*, as did Heilbrunn and Daugherty (1931) in *Amoeba dubia*. Cholodnyj (1923) found potassium and lithium produce marked increase in protoplasmic viscosity in root hairs of *Trianea* but that calcium and magnesium have no appreciable effect. Weber (1924) reached similar conclusions in reference to effects of sodium and calcium chloride on *Spirogyra*, and Spek (1921) maintains that viscosity of the protoplasm in *Actinosphaerium* is increased by cations in the following order: Li > K > Na > Ca > Mg. Spek (1920) found LiBr, LiCl, and KOH cause *Paramecium* to increase in volume, and he attributes this to a swelling action of the colloid resulting in increased viscosity. There is consequently, considerable evidence which indicates that monovalent cations induce increase and bivalent cations decrease in viscosity of protoplasm.

If this is true and the cilia are under control of the neuromotor apparatus, it is conceivable that an increase in viscosity of the cytoplasm could produce impulses in the neuromotor apparatus resulting in reversal in the action of the cilia.

That reversal is associated with increased viscosity is further suggested by the observation that reversal in response to temperature occurs only at temperatures almost immediately lethal, it being well established that there is a marked increase in viscosity preceding death at high temperatures. It is also shown that duration of reversal induced by chemicals varies inversely with temperature, and there are many observations which indicate that viscosity of protoplasm varies inversely with temperature

Precisely how environmental changes which have been observed to induce reversal in the action of cilia effect reversal and the physiological factors involved cannot be completely explained on a basis of our present knowledge. Factors such as changes in the electric potential at the surface, or in permeability and consistency of the surface layer of the organism, or chemical changes in the receptors or elsewhere in the organism may be involved as has been suggested. It is believed, however, that there is considerable evidence to indicate that reversal is associated with increase in viscosity of the cytoplasm. A more comprehensive study of this problem is being undertaken.

SUMMARY

1. The chlorides, bromides, and iodides of potassium, lithium, sodium, and ammonium induce reversal in the direction of the effective beat of the cilia of *Paramecium*. The chlorides, bromides, and iodides of calcium and magnesium do not.

2. The duration of reversed ciliary action varies directly with concentration of the salt which induces it.

3. The effect of monovalent salts on reversal is primarily due to the action of the cations. If the anions have any effect, it is quantitative and is secondary to that of the cations.

4. The order of effectiveness of the monovalent cations in inducing reversal is K > Li > Na > NH_4.

5. Paramecia which have been 18 hours in a given concentration of chloride, bromide, and iodide of potassium, lithium, and sodium become adapted and do not exhibit ciliary reversal when transferred to solutions of lesser concentration but do reverse to solutions of higher concentration. Duration of reversal in adapted is less than in nonadapted specimens. Ciliary reversal is dependent upon rate of increase in concentration of monovalent cation salt in the environment.

6. The order in which the monovalent cations produce death in *Paramecium* is Li > K > Na.

7. Prolonged exposure to M/40 lithium and potassium salts frequently results in change in direction of rotation on the longitudinal axis in forward locomotion and active changes in shape during locomotion.

8. Change in temperature which is not lethal does not induce reversal in direction of the effective beat in the cilia in *Paramecium*.

9. Lethal temperatures induce reversal in the action of the cilia if death is not immediate, but the duration of the reversed action is very short.

10. The duration of reversed ciliary action in solutions of monovalent cation salts varies inversely with temperature.

11. The order of effectiveness of monovalent cations in inducing reversal is independent of temperature.

12. The rate at which the cilia of paramecia beat in reverse varies directly with temperature.

LITERATURE CITED

CHOLODNYJ, N. 1923. Zur Frage über die Beeinflussung des Protoplasmas durch mono und bivalente Metallionen. Beihefte Bot. Centralbl., **39**: 231.

EISENBERG-HAMBURG, E. 1932. Einfluss der Sr-Salze auf die Bewegung von *Paramecium caudatum*. Arch. f. Protist'kunde, **77**: 108.

HEILBRUNN, L. V. 1923. The colloid chemistry of protoplasm, I: General considerations; II: The electrical charges of protoplasm. Amer. Jour. Physiol., **64**: 481.

HEILBRUNN, L. V., and DAUGHERTY, K. 1931. The action of sodium, potassium, calcium, and magnesium on the protoplasm of *Amoeba dubia*. Physiol. Zoöl., **4**: 635.

JENNINGS, H. S. 1899*a*. Studies on reactions to stimuli in unicellular organisms, II: The mechanism of the motor reactions of *Paramecium*. Amer. Jour. Physiol., **2**: 311.

———. 1899*b*. Studies on reactions to stimuli in unicellular organisms, IV: Laws of chemotaxis in *Paramecium*. *Ibid.*, 355.

MASSART, J. 1891. Recherches sur les organismes inférieurs. La Sensibilité à la concentration chez les êtres unicellulaires marins. Bull. Belg. Acad., Ser. 3. **22**: 148.

MAST, S. O. 1911. Light and the behavior of organisms. New York. Pp. 410.

MAST, S. O., and NADLER, J. E. 1926. Reversal in ciliary action in *Paramecium caudatum*. Jour. Morph. and Physiol., **43**: 105.

MERTON, H. 1923. Studien über Flimmerbewegung. Arch. f. d. ges. Physiol. **198**: 1.

———. 1935. Zwangsreaktionen bei *Stentor* als Folge bestimmter Salzwirkungen. Biol. Zentrabl., **55**: 268.

PARKER, G. H. 1896. The reactions of *Metridium* to food and other substances. Bull. Mus. Comp. Zoöl., **29**: 107.

———. 1905*a*. The reversal of ciliary movement in metazoans. Amer. Jour. Physiol., **13**: 1.

———. 1905*b*. The reversal of the effective stroke of the labial cilia of sea anemones by organic substances. *Ibid.*, **14**: 1.

PITTS, R. F. 1932. The relation between hydrogen ion concentration, inorganic salt concentration, and rate of locomotion in *Amoeba proteus*. Johns Hopkins University dissertation. Pp. 191.

SPEK, J. 1920. Experimentelle Beiträge zur Kolloid-Chemie der Zellteilung. Kolloidchem. Beihefte, **12**: 1.

———. 1921. Der Einfluss der Salze auf die Plasmokolloide von *Actinosphaerium Eichorni*. Acta. Zool. (Stockholm), **2**: 153.

STRASBURGER, E. 1878. Wirkung des Lichtes und der Wärme auf Schwärmsporen. Jena Zeitsch., N.F., **12**: 551.

WEBER, F. 1924. Plasmolyseform und Protoplasmaviskosität. Österreich Bot. Zeitsch., **73**: 261.

THE INFLUENCE OF CERTAIN PLANT HORMONES ON GROWTH OF PROTOZOA[1]

(Six figures)

ALFRED M. ELLIOTT
Biological Laboratories, Stanford University, and State Teachers College, Bemidji, Minnesota

IT IS generally agreed that growth of stems and leaves of higher plants is stimulated by plant hormones, while growth of roots, saprophytic plants (e.g., bacteria, yeasts, and molds), and animal cells is either unaffected or else retarded in some cases. Whether this difference in effect is dependent upon some definite correlation between the presence of chlorophyll and the activity of phytohormones is not yet known, although the available evidence favors such an assumption.

The possibility that there might be some intermediate point between typical plant and animal cells at which the phytohormones lose their stimulating effect suggested a comparison of the influence of several synthetic phytohormones on the growth of three protozoa whose nutritional requirements are fairly well known. The three species selected—the chlorophyll-bearing *Euglena gracilis; Khawkinea halli*, without chlorophyll but otherwise similar to *Euglena* to which it is related; and the ciliate, *Colpidium striatum*—represent adaptations to three different methods of nutrition: holophytic. saprozoic, and holozoic, respectively. If the accelerating effects of phytohormones are dependent upon presence of chlorophyll, there should be no such effect in the case of *C. striatum* or even with *K. halli*.

MATERIAL AND METHODS

The three species of protozoa used in this investigation were maintained in bacteria-free cultures, purity being determined by periodic bacteriological tests. The strains of *E. gracilis* and *K. halli* were obtained from Professor R. P. Hall, the latter having been isolated originally by Dr. T. L. Jahn and designated previously as *Astasia* sp. (Jahn and McKibben, 1937). The strain of *C. striatum* was isolated several years ago by the writer (Elliott, 1933).

The following plant hormones were obtained from the Eastman Kodak Company: γ-3-n-indolebutyric acid ($C_{12}H_{13}O_2N$), β-3-indolepropionic acid ($C_{11}H_{11}O_2N$), and 3-indoleacetic acid ($C_{10}H_9O_2N$), or heteroauxin. Solutions of these substances were prepared a few hours before use in order to insure maximal activity.

As a basic organic medium for *C. striatum*, Difco "tryptone" was made up in 2.0 per cent solution, a concentration near the optimum for this ciliate. For the flagellates,

[1] This investigation was supported in part by grants from the Minnesota Academy of Science and the American Association for the Advancement of Science.

The writer wishes to express his gratitude to Professor C. V. Taylor, in whose laboratory the preliminary experiments were performed; to Dr. P. W. Zimmerman, who suggested the three indole compounds employed in the study; to Professor R. P. Hall, who carefully read the manuscript; and to Mr. Anthony Rozycki for technical assistance.

Euglena and *Khawkinea*, the same peptone was used in 0.5 per cent concentration in the following solution:

NaCl	0.003 gm.	$MgSO_4$	0.005 gm.
KNO_3	0.002	$FeSO_4$	Trace
$CaSO_4$	0.020	Glass distilled water	1.0 liter

In determining the effects of phytohormones in relation to pH, suitable quantities of each basic medium were adjusted to the desired pH, tubed in 8.0-cc. amounts, and then sterilized in the autoclave at 15 pounds pressure for 20 minutes. The indole solutions were made up in glass distilled water and sterilized in the autoclave for 30 minutes at 115° C. To each experimental tube 1.0 cc. of sterile indole solution was added aseptically, while the controls each received 1.0 cc. of sterile distilled water. Both experimental and control tubes were inoculated with 1.0-cc. suspensions of the organisms to be tested. The flasks from which the inoculations were made were prepared from an actively growing tube of organisms, previously cultured for several weeks and through several transfers in tryptone medium. The organisms were washed and centrifuged before addition to the flask in order to remove most of the fluid in which they were growing.

Initial pH determinations were made with representative control and experimental tubes after inoculation. The initial concentration of organisms in each series was determined by counts on samples from the stock flask, the count per cubic centimeter in the inoculated tubes then being computed. In every series the cultures were incubated at a constant temperature of 26° C. The preliminary series were maintained in a constant temperature room at Stanford University (accuracy, ± 0.1° C.) while the confirmatory series were run in a constant temperature bath at State Teachers College (accuracy, ± 0.02° C.). In the case of *Euglena* the cultures were placed at 14 inches from a continuous source of artificial illumination (100-watt electric lamp). After incubation the final pH of two or more tubes from each set was determined and the remainder were fixed for counting by the addition of 0.5 cc. of a saturated solution of trinitrophenol in 1N HCl. As described previously (Elliott, 1933), all counts were made with a Sedgwick-Rafter counting chamber and a Whipple ocular micrometer. A LaMotte colorimeter was used for pH determinations. In experiments testing the effect of different concentrations of indole compounds on the growth of *Euglena*, it was necessary to culture the flagellates in 250-cc. Erlenmeyer flasks instead of tubes. Two cubic centimeter samples were withdrawn from the flasks at regular intervals and counted.

EFFECTS OF PHYTOHORMONES IN RELATION TO pH

Since it has already been noted (Elliott, 1935*a*) that pantothenic acid accelerates growth of *C. striatum* below pH 7.0 and yet is actually toxic above the neutral point, it seemed necessary to determine whether or not the effects of phytohormones might also depend upon the pH of the medium. Consequently, tests were made over the pH range, 3.0–9.0.

In the preliminary experiments it was necessary to start with an arbitrarily selected concentration of plant hormones. The concentration first used, 1:10,000 in Series I*a* and II*a*, proved toxic and subsequent tests showed that a dilution of 1:200,000 or higher was satisfactory for stimulation of *Euglena*. Hence, dilutions of 1:200,000 were used in Series I*b*, II*b*, and III.

Series Ia (*C. striatum*).—Each indole compound was added to the basic medium in concentration of 1:10,000. The pH of different sets of experimental and control tubes was

adjusted as follows: 3.5, 4.5, 5.5, 6.8, 7.6, and 8.8. The initial count was 125 ciliates per cubic centimeter. After incubation for 48 hours the pH was unchanged except for a rise of 0.2 in the tubes with an initial pH of 5.5 and 6.8. Such shifts were too small to influence the results significantly.

The final counts (Fig. 1) showed that no acceleration was produced by the phytohormones. Indoleacetic acid inhibited growth almost completely at all pH levels and no attempt was made to plot the results. Indolepropionic acid was slightly toxic at pH 5.5, while indolebutyric acid produced a decided decrease in growth at the same point. At pH 6.8 and 7.6 the former showed no significant influence while the latter showed a slightly depressed effect. The results show clearly that the inhibitory effect of these two indole compounds is much greater below pH 7.0 than above the neutral point, a correlation comparable to that reported previously (Elliott, 1935*b*) for sodium acetate and sodium butyrate.

Series Ib (*C. striatum*).—In this series the growth substances were used at a concentration of 1:200,000 in tryptone solutions at pH 3.8, 4.7, 5.7, 6.8, 7.5, and 9.0. The initial count was 200 ciliates per cubic centimeter. After incubation for 45 hours pH changes were comparable to those in Series I*a*.

The final counts (Fig. 2) showed that indolepropionic and indolebutyric acid had produced practically the same effects as in Series I*a*, while indoleacetic acid was somewhat more toxic than indolepropionic acid. Here again the effects were less pronounced above pH 7.0.

Later experiments with higher dilutions (1:10,000,000) showed results similar to those of Series I*a* and I*b*, except that the inhibitory effect of the growth substances was much less striking, as might be expected. It is clear that, within the limits of these experiments, growth of *C. striatum* is not accelerated by heteroauxin, indolepropionic acid, and indolebutyric acid. It would seem, therefore, that these growth substances, which bring about such striking responses in higher plants, are not essential in the normal growth of this ciliate protozoan.

The bimaximal growth curve so typical of *C. striatum* in tryptone medium (Elliott, 1933) was absent in both Series I*a* and I*b*. Preliminary observations suggest that this may be due to the omission of the phosphate buffer from the basic medium used in the present investigation.

Series IIa (*K. halli*).—The indole compounds were used in concentrations of 1:10,000 in the 0.5 per cent tryptone medium at pH 3.2, 4.5, 5.5, 6.5, 7.5, and 9.0. The initial count was 400 flagellates per cc. No appreciable changes in pH had occurred after a 7-day incubation period. The final counts (Fig. 3) showed that the indole compounds failed to accelerate growth at any pH, and that indoleacetic acid was highly toxic in the concentration used.

Series IIb (*K. halli*).—In this series the concentration of growth substances was reduced to 1:200,000, and the initial pH of the different sets of tubes was as follows: 3.2, 4.5, 5.5, 6.7, 7.4, and 8.8. The initial count was 630. Cultures were incubated for 7 days and then fixed for counting. Final counts (Fig. 4) again failed to show an acceleration of growth, although the inhibitory effects were less marked than in Series II*a*. Comparable experiments with the hormones in dilutions of 1:10,000,000 likewise showed no acceleration of growth. It seems certain, therefore, that the indole compounds tested do not accelerate growth of *K. halli*.

Series III (*E. gracilis*).—In view of the previous experience with *Colpidium* and *Khawkinea* the phytohormones were used at a concentration of 1:200,000 in 0.5 per

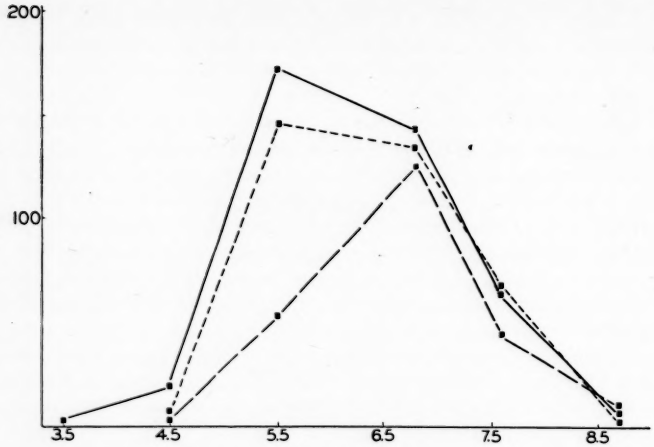


FIG. 1.—Series I*a*. Concentration of ciliates in thousands per cubic centimeter are plotted against initial pH. Cultures are indicated as follows: Control (——), indolepropionic acid (- - - - -) and indolebutyric acid (— —). Concentration of indole compounds is 1:10,000.

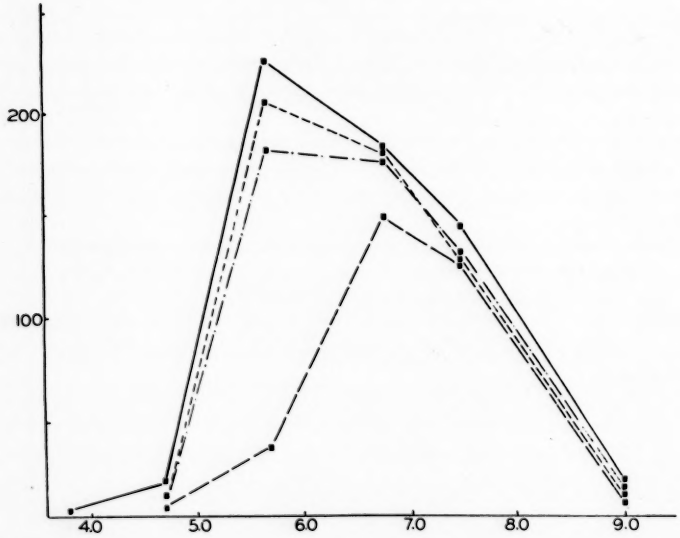


FIG. 2.—Series I*b*. Concentrations of ciliates in thousands per cubic centimeter are plotted against initial pH. Cultures are indicated the same as in Fig. 1 and indoleacetic acid (— · —) is added. The indole compounds have a concentration of 1:200,000.

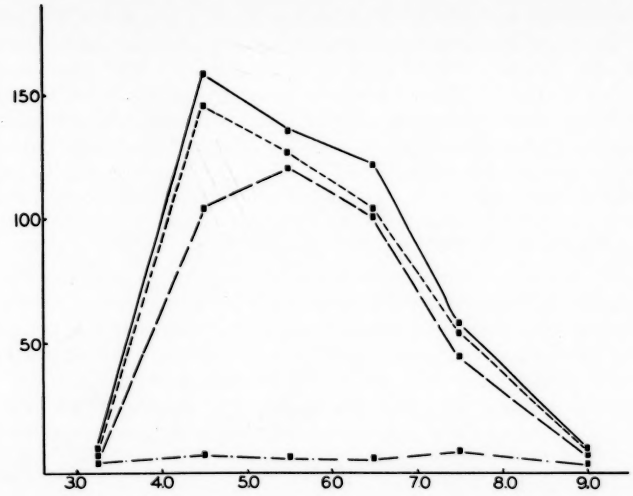


FIG. 3.—Series II*a*. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. Cultures are designated the same as in Fig. 2. Concentration of indole compounds is 1:10,000.

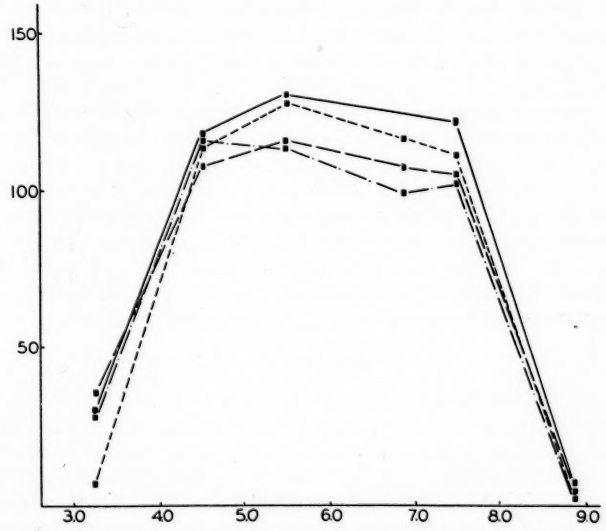


FIG. 4.—Series II*b*. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. Cultures are indicated as in Fig. 2. Concentration of indole compounds is 1:200,000.

cent tryptone medium with initial pH as follows: 3.0, 4.5, 5.5, 6.6, 7.5, and 9.0. The initial count was 3,300 flagellates per cubic centimeter. No appreciable changes in pH had occurred after incubation for 6 days under constant artificial illumination.

The final counts (Fig. 5) showed acceleration of growth by indolebutyric acid over the entire pH range, and especially at pH 5.5. Indolepropionic acid produced significant acceleration at pH 5.5 but little or no effect at the other pH levels. Heteroauxin showed little or no effect over the pH range tested; as determined later (Series IV) this was due to the fact that a concentration of 1:200,000 is too high for this growth substance.

EFFECTS OF DIFFERENT CONCENTRATIONS OF PHYTOHORMONES

Series IV (*E. gracilis*).—Since acceleration of growth was pronounced only in the case of *E. gracilis*, experiments to determine the effects of different concentrations of indole compounds were limited to this species. The different indole compounds were added to flasks of 0.5 per cent tryptone medium in concentrations of 1:10,000, 1:100,000, 1:1,000,000, and 1:10,000,000. The initial pH in each flask was 5.6, and the initial count was 1,400 flagellates per cubic centimeter. The flask cultures were incubated under constant illumination for 9 days, 2.0-cc. samples being removed asceptically from each flask at 2-day intervals for determining the concentration of flagellates. The results (Fig. 6) show that growth is accelerated by indolepropionic acid in the three higher dilutions but not at 1:10,000. Indolebutyric acid showed marked acceleration of growth at dilutions of 1:1,000,000 and 1:10,000,000, but not in the highest concentration used. A decided acceleration was produced by indoleacetic acid in a concentration of 1:10,000,000; no effect was produced by 1:100,000, while growth was definitely retarded in a 1:10,000 dilution. No effort was made to plot the numbers of organisms beyond the seventh day of growth because counting became too difficult. However, counts on the ninth day with the "cytocrit"[2] indicated the same sort of relative growth as was observed on the seventh day. In all cases acceleration of growth, when it occurred, was obvious on the fourth day of growth. Other observations, not described here in detail, showed that the most effective concentration of indoleacetic acid lies between 1:1,000,000 and 1:10,000,000. These observations are in accord with the findings of Zimmerman and Wilcoxson (1935), who found indoleacetic acid to be five times as powerful as indolepropionic acid and indolebutyric acid in inducing responses in higher plants.

Series V (*E. gracilis*).—The same procedure was followed as in Series IV, except that the initial pH was 7.4 in all cases instead of 5.6. The results indicated that all three compounds were more effective in concentrations of 1:100,000–1:1,000,000 than in higher and lower dilutions in stimulating growth of *Euglena*. The accelerating effects were always slight, however, as compared with those observed at pH 5.6.

DISCUSSION

Since Went (1927) discovered a method for extracting plant hormones and demonstrating their quantitative effects on growth in *Avena* coleoptiles, much has been accomplished in identifying other substances which cause similar responses in higher plants. Zimmerman and Wilcoxon (1935) have shown that a large number of chemically diverse synthetic compounds cause responses in higher plants similar to those produced by auxins *a* and *b*. These synthetic compounds incite such striking effects as development of roots on leaves and stems, bending of stems, acceleration of growth, and epinasty

[2] A specially designed centrifuge tube used in estimating volume of packed protozoa in determining relative growth. This will be described in detail in a later publication.

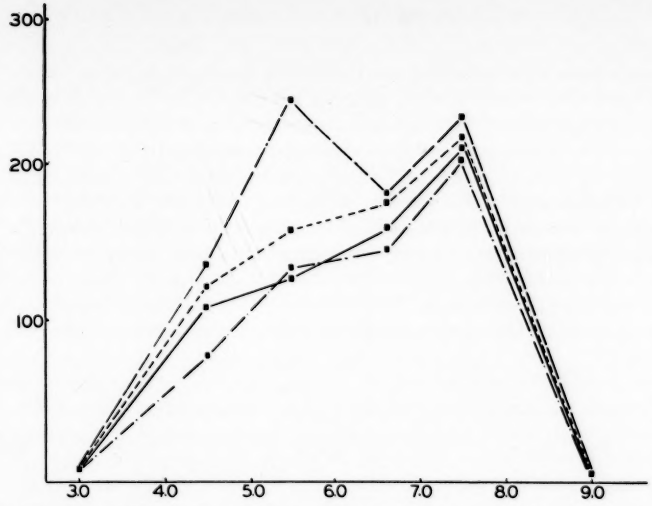


FIG. 5.—Series III. Concentration of flagellates in thousands per cubic centimeter plotted against initial pH. Cultures are designated the same as in Fig. 2. Concentration of indole compounds is 1:200,000.

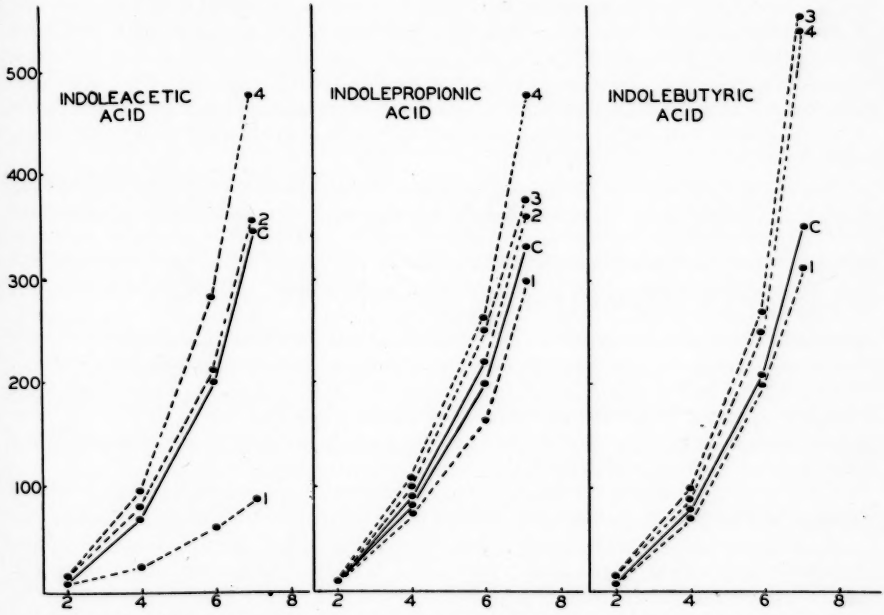


FIG. 6.—Series IV. Effect of different concentrations of phytohormones. Concentration of flagellates in thousands per cubic centimeter plotted against time in days. The dashed lines indicate cultures containing the indole compounds in concentrations indicated as follows: 1 = 1:10,000; 2 = 1:100,000; 3 = 1:1,000,000; 4 = 1:10,000,000. The solid lines are the controls in each case.

of leaves. Since such compounds produce profound changes in higher plants it might be expected that they would exert analogous effects on chlorophyll-bearing protozoa. Popoff (1933) demonstrated that low concentrations of growth substances extracted from coleoptiles and other parts of *Zea* seedlings accelerated cell division and excystment of *E. gracilis*. The purified indole compounds employed in this investigation also augmented multiplication of *E. gracilis*, indicating a marked influence on the rate of metabolism. The occurrence of autotrophic nutrition in *E. gracilis*, described by Dusi (1933) and earlier workers, has recently been questioned by Hutner (1936). If Hutner's findings can be corroborated, it may be inferred that *E. gracilis* shows a distinct tendency toward saprophytic nutrition. Nevertheless, this euglenoid flagellate still retains the property of utilizing the same substances which increase cell proliferation in higher plants.

It has been demonstrated that growth of lower plants, such as *Aspergillus niger*, is retarded by auxins, although such substances are produced in the tissues of the fungus (Boysen Jensen, 1932). Thus, auxins are found in fungi, yeasts, and bacteria, but usually fail to accelerate growth of the organism from which they are extracted. Whether or not *K. halli* elaborates such growth substances is not known, but it is obvious that growth was not accelerated by heteroauxin, indolepropionic, and indolebutyric acid. In this respect the flagellate resembles *A. niger*. The fact that *Khawkinea* is closely related to *Euglena*—the chief difference being the lack of chloroplasts in the former—reveals an apparent correlation between the presence of chlorophyll and the activity of plant hormones in Protozoa. That this flagellate is not photosynthetic has been demonstrated by Pringsheim (1921), who showed that it could not utilize ammonium compounds and single amino acids as sources of nitrogen. The change to saprophytism with loss of chlorophyll in this flagellate has been accompanied by a loss of the power to utilize growth substances which accelerate cell division of chlorophyll-bearing organisms.

These growth substances are found in animal cells and particularly in excretions such as urine. It is uncertain, however, that the auxins are synthesized by animal cells since they may possibly be derived from plants taken in as food. The available evidence indicates that these substances are not essential to growth of animal tissues. It has been shown, for instance, that growth of fibroblasts in tissue culture is not stimulated by auxins *a* and *b* (Kögl, Haagen Smit, and Tonnis, 1933). Likewise, the rate of metamorphosis of tadpoles is unaffected by auxin (Kögl, Haagen Smit, and Erxleben, 1933). Navez and Kropp (1934) failed to observe activity of the chromatophores of *Palaemonetes* when auxin was applied to the eyestalks. Greenberg and Schmidt (1936) observed no increase in growth of *Planaria maculata* when supplied with two different preparations of auxin. In view of the negative findings already obtained with animal cells, the present results with *Colpidium* were not surprising.

The effects of the indole compounds tested are interesting in comparison with results obtained in the case of pantothenic acid (Elliott, 1935*a*). With the latter, growth of *C. striatum* was markedly accelerated, but there was no effect on growth of the chlorophyll-bearing flagellate, *Haematococcus pluvialis*. With the indole compounds, the situation is reversed, no acceleration of growth being produced in the absence of chlorophyll.

The influence of hydrogen-ion concentration on the growth-promoting or inhibiting powers of the phytohormones showed a definite relationship. In those experiments where no acceleration was observed (Series I*a*, I*b*, II*a*, II*b*) the indole compounds were toxic throughout the entire pH range, but particularly so in the range of optimum growth

(pH 4.5–6.0), with the exception of indoleacetic acid in Series I*a* and II*a* where it was extremely toxic at all pH levels. Above pH 7.0, even in concentrations of 1:10,000 of the indole compounds, toxic effects were much reduced over those on the acid side of neutrality. Furthermore, in Series III and IV acceleration of growth of *Euglena* occurred with the indole compounds at the points of optimum pH for growth (pH 4.5–6.0). It seems apparent, then, that if the indole compounds augment growth of a protozoan it must be done at the point of optimum growth for that organism. Substances which augment or inhibit growth of the three species of protozoa studied here do it at approximately the same pH level. This probably has some relationship with the ionization of the acids involved.

SUMMARY

Three plant hormones—3-indoleacetic acid (heteroauxin), β–3–indolepropionic acid, and γ-3-indolebutyric acid—were tested for their effect as growth stimulants on three species of Protozoa in pure cultures. Comparative studies were made with a chlorophyll-bearing flagellate, *E. gracilis*, a colorless euglenoid flagellate, *K. halli*, and a holotrichous ciliate, *C. striatum*. The compounds were tested over a wide pH range (3.0–9.0) and it was demonstrated that in the case of *Euglena* growth was markedly accelerated with all three indole compounds at pH 5.6 and a concentration of 1:1,000,000 and 1:10,000,000. No favorable effects were noted with either *Khawkinea* or *Colpidium*, and at certain concentrations of the plant hormones retardation of growth was observed.

LITERATURE CITED

Boysen Jensen, P. 1932. Über die Bildung und biologische Bedeutung des Wachstumsregulators bei *Aspergillus niger*. Biochem. Zeitschr., **250**:270–80.

Dusi, H. 1933. Recherches sur la nutrition de quelques Euglenes. I. *Euglena gracilis*. Ann. l'Inst. Pasteur, **50**:550–97.

Elliott, A. M. 1933. Isolation of *Colpidium striatum* Stokes in bacteria-free cultures and the relation of growth to pH of the medium. Biol. Bull., **65**:45–56.

———. 1935*a*. The influence of pantothenic acid on growth of protozoa. Biol. Bull., **68**:82–92.

———. 1935*b*. Effects of certain organic acids and protein derivatives on the growth of *Colpidium*. Archive f. Protistenk., **84**:472–94.

Greenberg, L. D., and Schmidt, C. L. A. 1936. Studies on the properties of a growth-promoting factor for *Planaria maculata*. Jour. Exper. Zoöl., **73**:375–92.

Hutner, S. H. 1936. The nutritional requirements of two species of *Euglena*. Archive f. Protistenk., **88**:93–106.

Jahn, T. L., and McKibben, W. R. 1937. A colorless Euglenoid flagellate *Khawkinea halli*, n. gen., n. sp. Trans. Am. Micr. Soc., **54**:48–54.

Kögl, F.; Haagen Smit, A. J.; and Erxleben, H. 1933. VII. Mitteilung. Studien über das Vorkommen von Auxinen im menschlichen und im tierischen Organismus. Hoppe-Seyl. Zeitschr. Phys. Chem., **220**:137–61.

Kögl, F.; Haagen Smit, A. J.; and Tonnis, B. 1933. VIII. Mitteilung. Über das Vorkommen von Auxinen und von Wachstumsstoffen der "Bios"-Gruppe in Carcinomen. Hoppe-Seyl. Zeitschr. Phys. Chem., **220**:162–72.

Navez, A. E., and Kropp, B. 1934. The growth promoting action of crustacean eye-stalk extract. Biol. Bull., **67**:250–58.

Popoff, M. 1933. Über die pflanzlichen Auxine und ihre Wirkung auf Einzellige. Biol. Zentralbl., **53**:661–68.

Pringsheim, E. G. 1921. Zur Physiologie der saprophytischen Flagellaten (*Polytoma*, *Astasia* und *Chilomonas*). Beitr. z. allg. Bot., **2**:88–138.

Went, F. W. 1927. On growth-accelerating substances in the coleoptile of *Avena sativa*. Proc. K. Akad. Wetensch. Amsterdam, **30**:10–19.

Zimmerman, P. W., and Wilcoxon, F. 1935. Several chemical growth substances which cause initiation of roots and other responses in plants. Contr. Boyce Thompson Inst. Plant Res., **7**:209–29.

SOME EFFECTS OF HYPERTONIC SOLUTIONS UPON DEVELOPMENT AND OXYGEN CONSUMPTION[1]

(Twelve figures)

ARTHUR LOUIS SCHIPPER
Zoölogical Laboratory, State University of Iowa

INTRODUCTION[2]

CONSIDERABLE attention has been accorded the changes in water content of animal cells and tissues. Aquatic forms have served as experimental material for many of these investigations. The methods by which aquatic animals control the intake and outgo of water, together with the expenditure of energy necessary to maintain this water balance, has been thoroughly analyzed by Schlieper (1930, 1935). The role which proteins play in regulating the movements of water in living organisms, plus the oxygen consumption concerned in this regulation, has been emphasized by Jordan Lloyd (1933). Buxton (1932), investigating the humidity of the environment and terrestrial insects, and Mellanby (1935), examining the evaporation of water from insects, in extensive reviews have pointed out the physiological mechanisms governing water exchange.

Hypertonic solutions bring about alterations of the water content of animals and so produce changes in the weight (Adolph, 1936; Thompson and Bodine, 1936), volume (Kitching, 1934; Slifer, 1934; Thompson and Bodine, 1936), and oxygen consumption (Borei, 1935; Bodine, 1933, 1934; Thompson and Bodine, 1936).

Although the water content of insects under various conditions has been extensively studied, most observations have been on other than egg and embryonic stages. Bodine (1929) compared the water content and rate of oxygen consumption of developing *Melanoplus differentialis* eggs. The same author (1933, 1934) and, more recently, Thompson and Bodine (1936) have shown that hypertonic solutions depress the rate of oxygen consumption. Slifer (1934) reported that the resistance of postdiapause embryos to hypertonic solutions is dependent upon the concentration and the length of exposure. The purpose of this investigation has been to determine the effects of dehydration and dehydration-rehydration on cellular activity by noting viability, extent of development, and changes in the rate of oxygen consumption.

MATERIALS AND METHODS

Approximately 40,000 eggs of the grasshopper, *M. differentialis* (Thomas), have been used during the course of this investigation. The early stages (prediapause) have been designated as 1-, 5-, 10-, and 15-day eggs. After 17 or 18 days there is an interruption of development, diapause, which separates the pre- and postdiapause stages. Diapause is a naturally occurring period of inactivity concurrent with a low rate of oxygen consump-

[1] Aided by a grant from the Rockefeller Foundation for Research in Cellular Physiology.

[2] The writer wishes to express his appreciation to Professor J. H. Bodine for the manifest interest and many helpful suggestions rendered during the progress of this investigation.

tion and little change in water content. Eggs, collected on the day they were laid, were transferred to a constant-temperature chamber and kept there until the desired stage of development had been attained. (All eggs, control and experimental, were kept at 28° C.) Postdiapause eggs were obtained by subjecting diapause eggs to a low temperature (5° C.) for two months and then returning them to 28° C. The morphological stages have been described by Slifer (1932*a*, 1932*b*) and Slifer and King (1934). Bodine (1929) and Boell (1935) have determined the changes in the rate of oxygen intake during development.

Control eggs were kept on moist paper toweling while experimental ones were immersed in distilled water or in a balanced salt solution. Shell vials (1″ × 6″), containing 10 to 100 eggs in 25 cc. of solution, were suspended in a constant-temperature water bath. The solutions were changed daily.

The isotonic salt (modified Ringer's) solution was composed of 0.9 per cent NaCl and 0.02 per cent each of KCl and $CaCl_2$ and has been designated as N, the hypertonic solutions being designated accordingly (e.g., 5 N). Slifer (1934), using $NaHCO_3$ in addition to the above, found no adverse results when postdiapause embryos were placed in solutions ranging from 0.9 to 1.3 times the normal concentration.

Early in this investigation it was noted that the development of immersed eggs was retarded and eventually inhibited unless the solution containing the eggs was aerated. Aeration was effected by a continuous stream of air introduced into the solution through small-bore glass tubes; these aerating tubes were lined with a thin film of vaseline to prevent crystallization of the salts. Distributors, each with several taps, made possible the aeration of a large number of solutions simultaneously.

Modified Krogh microdifferential manometers, calibrated according to the method by Dixon (1934), were used in measuring changes in the rate of oxygen consumption. The number of eggs used in an experimental lot was dependent upon the stage of development attained, determinations being made with 20 prediapause, an equal number of diapause, and 10 postdiapause ones. The high rate of oxygen consumption of postdiapause eggs necessitated the use of fewer ones of this stage. Consecutive determinations for a single lot of eggs were made with the same manometer. Control eggs were placed in manometers with no solution while experimental eggs were placed in manometers containing 1 cc. of the solution to which the eggs had been exposed. The manometers were shaken while in the water bath to insure an equal distribution of oxygen dissolved in the solution. Each manometer cup was provided with a small well for KOH, 0.15 cc. of 2 per cent KOH being used to remove the carbon dioxide produced by the eggs.

EXPERIMENTAL RESULTS

DEVELOPMENT IN HYPERTONIC SOLUTIONS

The solutions employed in this investigation were 1, 2, 3, 4, 5, 7.5, 10, 15, and 20 N. One hundred 1-day eggs were placed in each of the solutions. Similar lots of eggs were placed in moist sand and in distilled water. The number of eggs developing was determined when the embryos were removed 20 days later (Fig. 1). More than 95 per cent of the eggs which had been in moist sand, distilled water, N, or 2 N solutions contained developing embryos; 85 per cent in 3 N, 50 per cent in 4 and 5 N, 25 per cent in 7.5 and 10 N and less than 10 per cent in 15 and 20 N. (The reader is referred to Table 1 for the development attained by these eggs.) Loss of water, as indicated by a loss of turgidity of the eggs, occurred in all hypertonic solutions. Some eggs in 5 N, and practically all

in 15 and 20 N solutions, were flattened. This flattening was caused by the removal of water from the eggs by the hypertonic solutions. Solidification of the yolk, resulting in disintegration of the embryos, was probably responsible for the small percentage of developing eggs in the higher concentrations.

The effects of dehydration upon the extent of embryonic development were determined by placing 80 1-day eggs in each of the solutions. Embryos were removed and examined from some of the eggs 10 days later and at 5-day intervals thereafter (Table 1).

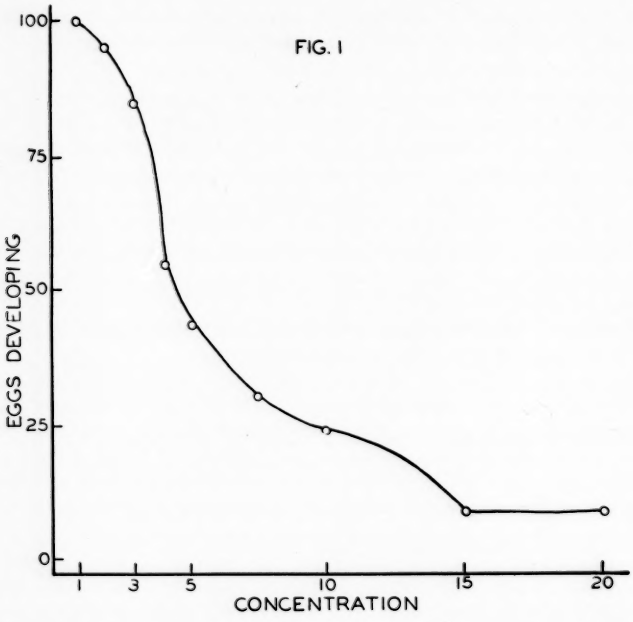


FIG. 1.—Eggs containing embryos after 20 days' exposure to solutions ranging from N to 20 N. Eggs were immersed on the day they were laid.

It is to be remembered that, with this retardation of growth, there was also a reduction in the number of eggs containing embryos (Fig. 1). This prolongation of the developmental period gradually merged into an inhibition of development; the stage at which cessation of growth occurred was dependent upon the hypertonicity of the solution. No embryos developed as far as the diapause stage in any of the hypertonic solutions.

Eighty postdiapause eggs were placed in each of the solutions on the day of removal from 5° C. A similar lot of eggs was kept on moist paper toweling. Hatching began in the control lot after 13 days and in N after 16 days. No hatching occurred in the hypertonic solutions, although many of the embryos had undergone considerable postdiapause development in concentrations ranging from 2 to 5 N. Beyond 5 N only a small number were able to initiate blastokinesis (Table 2). Reduction in size and disintegration of the embryos were frequently noted after exposures to hypertonic solutions.

Similar experiments were conducted with later stages of postdiapause eggs. Hatching was noted in 2 and 3 N if the postdiapause eggs were allowed to develop three days before being immersed in these solutions. If postdiapause eggs were allowed to develop six days before being dehydrated, hatching occurred in concentrations as great as 7.5 N. It is thus apparent that the eggs become more resistant to dehydration as postdiapause development continues.

TABLE 1*

EFFECT OF CONCENTRATION ON PREDIAPAUSE DEVELOPMENT

Length of Exposure (Days)	Concentration								
	N	2 N	3 N	4 N	5 N	7.5 N	10 N	15 N	20 N
10	10†	9	9	8	8	7	7	4	4
15	15	11	11	10	10	9	8	4	4
20	D‡	13	13	12	11	10	9	4	4
25	D	15	14	12	12	11	9	4	4
30§	D	15	14	13	13	12	10	4	4

* One-day eggs were placed in solutions; the embryos of a few eggs were removed and examined at the intervals indicated.
† Stage of embryo as compared to control at 28° C.
‡ Diapause.
§ General inhibition after this period.

TABLE 2*

EFFECT OF CONCENTRATION ON POSTDIAPAUSE DEVELOPMENT

	Concentration								
	N	2 N	3 N	4 N	5 N	7.5 N	10 N	15 N	20 N
Percentage Initiating blastokinesis	90	63	66	77	70	35	15	27	11
Development attained†	*H*	*L*	*L*	*E*	*E*	*B*	*B*	*B*	*B*

* First-day postdiapause eggs were placed in solutions; the embryos were removed after 30 days' exposure.
† *H* = hatching; *L* = late yolk engulfment; *E* = early yolk engulfment; *B* = blastokinesis.

DEVELOPMENT FOLLOWING DEHYDRATION-REHYDRATION

The effects of hypertonic solutions were determined by dehydrating the eggs for a period of time and then transferring them to N. In this experiment five stages of development (1-, 5-, 10-, 15-day, and diapause) and five concentrations (5, 7.5, 10, 15, and 20 N) were used. The eggs were dehydrated 5, 10, 15, and 20 days in 5, 7.5, and 10 N; 4, 8, 12, and 16 days in 15 and 20 N. Following transfer to N some of the eggs burst owing to an excessive intake of water. This was most frequently noted with 5-day eggs and, to a lesser extent, with other stages. Such eggs, when examined, were found to contain no living embryos; the yolk presented a curdled appearance. Most of the embryos were removed 20 days after the eggs had been transferred to N and the number of developing embryos was determined (Table 3). A few eggs were allowed to remain, since it had been

noted that the duration of the diapause condition was materially shortened by dehydration-rehydration. Hatching occurred in a few cases, being most frequent in those eggs which had been allowed to develop 15 days before being dehydrated. One-day eggs were quite resistant to 5 N except for long periods of immersion (20 days). The susceptibility increased with concentration and length of exposure. There was less recovery for 5-day eggs than for 1-day ones, the ability to overcome such treatment being determined by the concentration rather than by the length of dehydration. There was considerable recovery

TABLE 3

RECOVERY OF DEHYDRATED EGGS FOLLOWING REHYDRATION

CONCENTRATION

5 N			7.5 N			10 N			15 N			20 N		
M*	E†	%‡	M	E	%	M	E	%	M	E	%	M	E	%
1	5	83	1	5	68	1	5	32	1	4	89	1	4	77
1	10	95	1	10	56	1	10	21	1	8	33	1	8	65
1	15	95	1	15	33	1	15	10	1	12	15	1	12	21
1	20	46	1	20	23	1	20	18	1	16	21	1	16	9
5	5	80	5	5	43	5	5	53	5	4	5	5	4	7
5	10	76	5	10	40	5	10	19	5	8	3	5	8	16
5	15	78	5	15	37	5	15	16	5	12	23	5	12	2
5	20	56	5	20	40	5	20	27	5	16	7	5	16	3
10	5	100	10	5	85	10	5	93	10	4	55	10	4	52
10	10	78	10	10	86	10	10	74	10	8	48	10	8	23
10	15	79	10	15	75	10	15	90	10	12	63	10	12	28
10	20	81	10	20	85	10	20	73	10	16	47	10	16	13
15	5	98	15	5	85	15	5	86	15	4	94	15	4	73
15	10	82	15	10	44	15	10	81	15	8	58	15	8	60
15	15	95	15	15	88	15	15	85	15	12	66	15	12	35
15	20	90	15	20	78	15	20	75	15	16	76	15	16	15
D§	5	100	D	5	63	D	5	97	D	4	90	D	4	81
D	10	98	D	10	52	D	10	78	D	8	90	D	8	90
D	15	97	D	15	82	D	15	73	D	12	88	D	12	90
D	20	100	D	20	87	D	20	82	D	16	91	D	16	73

* Morphological stage when dehydrated.
† Length of exposure to hypertonic solution.
‡ Recovery following transfer to N. § Diapause.

of 10- and 15-day eggs following dehydration by 5, 7.5, and 10 N although a marked susceptibility of these stages to 15 and 20 N was noted. Neither concentration nor length of immersion had much effect on the resistance of diapause eggs. It appears that the diapause eggs are able to retain water more tenaciously against osmotic forces than prediapause ones.

THE SECRETION OF THE CUTICLE IN HYPERTONIC SOLUTIONS

Twenty 1-day eggs were placed in each of the solutions and allowed to develop for 10 days. The contents of the eggs were then removed and the presence of the cuticular

layers[3] was determined by chemical methods (Jahn, 1935). It was found impossible to prevent, or even delay to any extent, the secretion of either layer of the cuticule. The secretory function of the serosa cells is apparently little disturbed by hypertonic solutions despite the fact that the embryos are profoundly affected by them.

OXYGEN CONSUMPTION

The changes in the rate of oxygen consumption of developing *M. differentialis* eggs have been carefully measured by Boell (1935). In the present investigation somewhat higher rates were found, these being attributable to the higher temperature at which these experiments were conducted. Changes in the rate of oxygen intake of developing eggs, on moist paper toweling and immersed in distilled water and in N, are shown in Figures 2 (prediapause) and 3 (postdiapause). It will be noted that the control eggs pos-

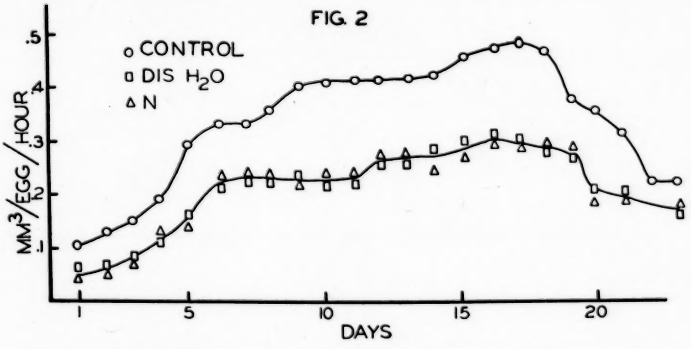


FIG. 2.—Changes in the rate of oxygen consumption of prediapause eggs

sessed a higher rate of oxygen consumption than the immersed ones. The differences in the rates were probably caused by the slower rate of diffusion of oxygen through the liquid medium.

The effects of hypertonic solutions on the oxygen consumption of 1-, 5-, 10-, 15-day, diapause, first-, and third-day post-diapause eggs have been investigated. Typical results are presented in Figures 4–8. It was frequently noted that the rate of oxygen consumption of a given stage of development was similarly affected by two or more concentrations. In such instances one concentration, together with the results produced by that concentration, was chosen to represent all those producing like results. Generally speaking, for the prediapause and diapause stages, there was but little difference in the rates of oxygen intake of these eggs in concentrations ranging from 3 to 10 N; likewise 15 and 20 N produced strikingly similar results (Figs. 4–6). A greater difference in the rates of oxygen consumption was noted for postdiapause eggs in hypertonic solutions (Figs. 7 and 8). The rate of oxygen consumption of eggs in the various hypertonic solutions was largely dependent upon the stage at which dehydration was started. These differences were most marked in those eggs which previously had had a high rate of oxygen intake (Fig. 8); likewise there was but little difference if the rate of oxygen consumption previous to immersion was low (Fig. 6).

[3] The histology of these layers has been described by Slifer (1937).

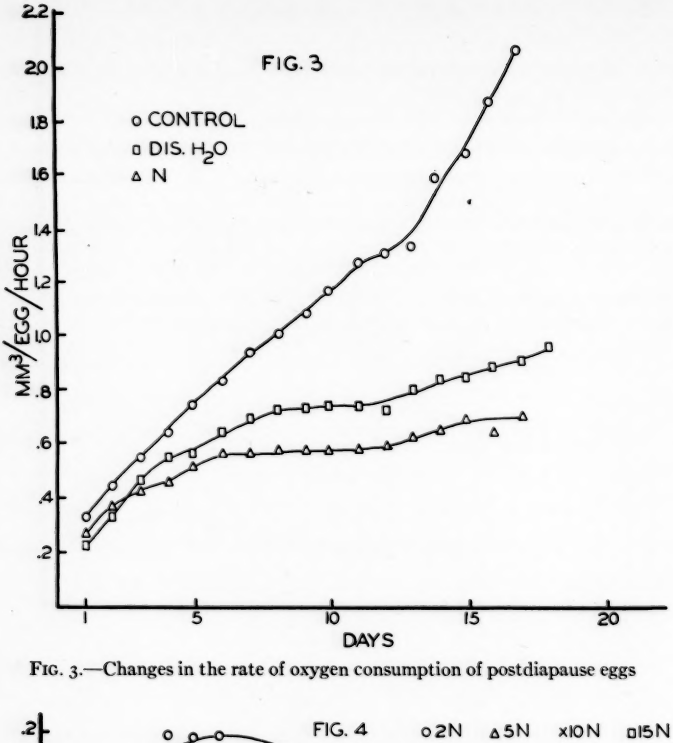
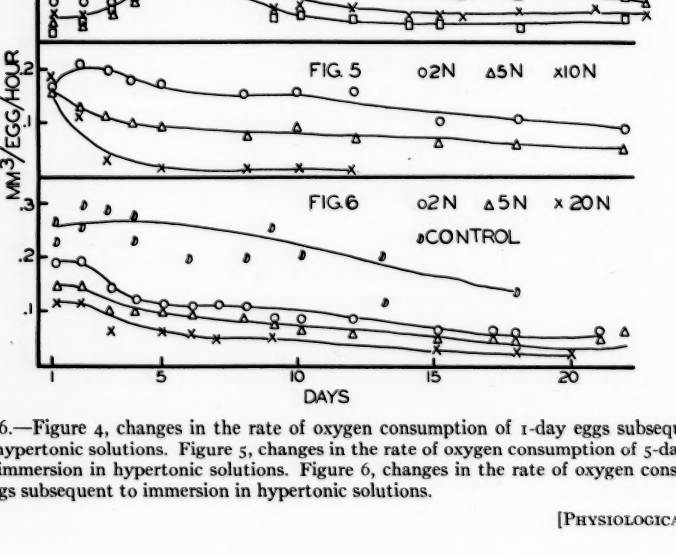
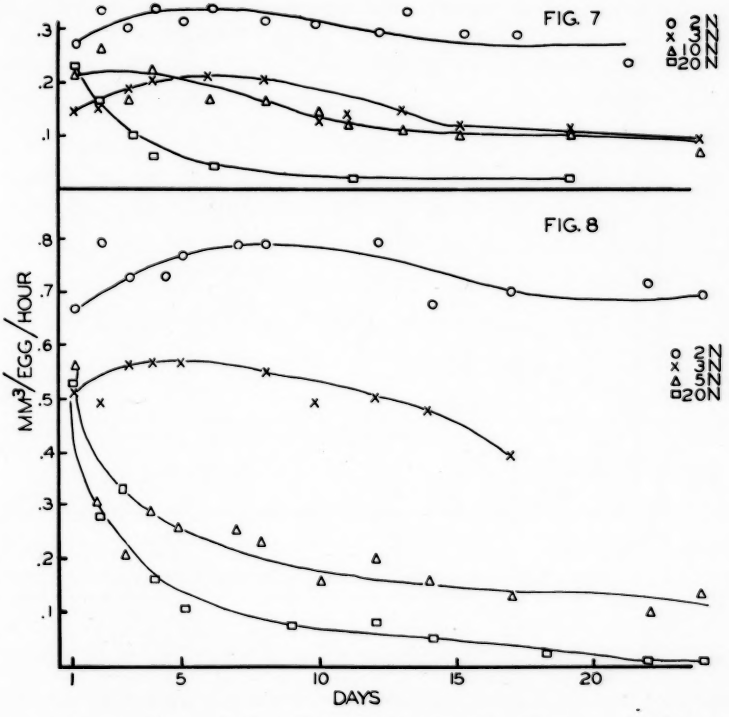


FIG. 3.—Changes in the rate of oxygen consumption of postdiapause eggs



FIGS. 4–6.—Figure 4, changes in the rate of oxygen consumption of 1-day eggs subsequent to immersion in hypertonic solutions. Figure 5, changes in the rate of oxygen consumption of 5-day eggs subsequent to immersion in hypertonic solutions. Figure 6, changes in the rate of oxygen consumption of diapause eggs subsequent to immersion in hypertonic solutions.

When 1-day eggs were placed in hypertonic solutions there was an initial increase in the rate of oxygen consumption, the peak being on the fifth and sixth days of exposure (Fig. 4); subsequent to the peak there was a decrease. As has been noted earlier in this investigation, embryos were formed in all concentrations, but further development depended upon the hypertonicity of the solution (Table 1). If the delay in development is compared with the reduction in the rate of oxygen consumption (Fig. 4), it will be noted that those solutions which cause the greater retardation also effect a corresponding depression of the oxygen intake.



FIGS. 7–8.—Figure 7, changes in the rate of oxygen consumption of first-day postdiapause eggs subsequent to immersion in hypertonic solutions. Figure 8, changes in the rate of oxygen consumption of third-day postdiapause eggs subsequent to immersion in hypertonic solutions.

When any of the following stages, 5-, 10-, 15-day, and diapause, were placed in hypertonic solutions there was an immediate decrease in the rate of oxygen consumption, the rate later tending to become constant. The changes in the rate of oxygen consumption of 5-day and diapause eggs are shown in Figures 5 and 6. The amount and rate of the decrease in oxygen intake were dependent upon the physiological stage of the cells at the time when the eggs were subjected to the hypertonic solutions. This is well illustrated by the effects of 10 N on 5-day eggs (Fig. 5) and of 20 N on diapause (Fig. 6). The cells

of 5-day eggs are in a period of active differentiation while those of the diapause eggs are in a state of quiescence owing to a developmental block. There was a marked decrease in the rate of oxygen consumption of 5-day eggs in 10 N although only a slight one was noted for diapause eggs in 20 N. The effects of hypertonic solutions on the amount of oxygen consumed by 10- and 15-day eggs were intermediate between those found for 5-day and diapause eggs. A general retardation of development of the prediapause eggs in hypertonic solutions was noted at the end of the experiment.

The rate of oxygen consumption of postdiapause eggs in hypertonic solutions varied considerably, depending upon the concentration of the solution and the stage of development (Figs. 7 and 8). There was a marked difference in the rates of oxygen intake of

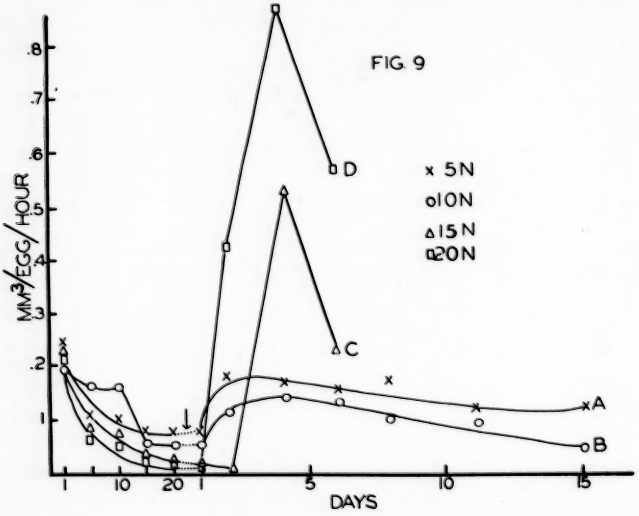
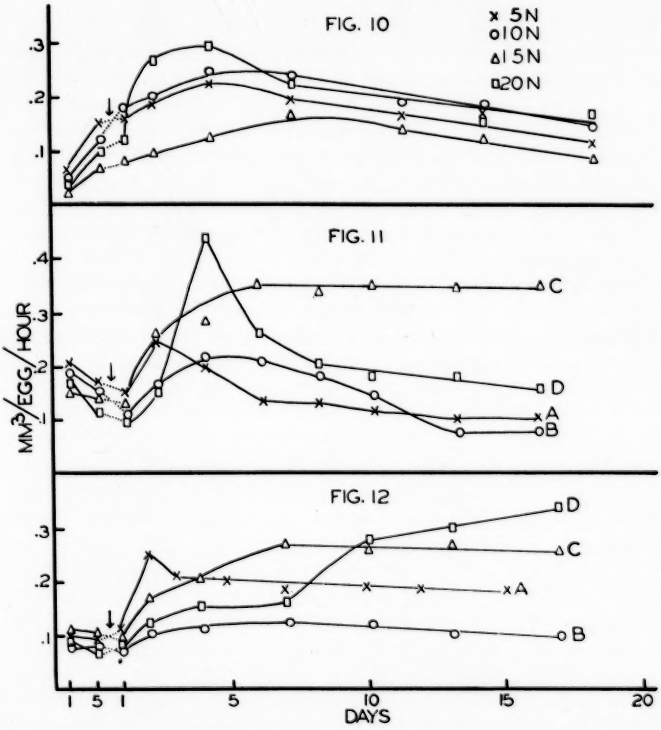


FIG. 9.—Changes in the rate of oxygen consumption of 15-day eggs immersed in hypertonic solutions for 20 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale.

postdiapause, diapause, and prediapause eggs in hypertonic solutions. This is to be noted when the rates of diapause eggs in 2, 5, and 20 N, as shown in Figure 6, and the rates of postdiapause eggs in 2, 3, and 20 N, as shown in Figures 7 and 8, are compared. It is to be remembered that postdiapause eggs have much higher rates of oxygen consumption than diapause ones. The differences in the rates of oxygen intake of postdiapause eggs in hypertonic solutions become much greater when later stages are dehydrated. The differences between the rates of oxygen consumption of postdiapause eggs in 2 and in 3 N is nearly as great as the difference between the rates of similar eggs in 3 and in 20 N. First-day postdiapause eggs in 3 and in 10 N consume oxygen at similar rates (Fig. 7) while 3-day postdiapause eggs in 3 and in 5 N utilize oxygen at widely separated rates (Fig. 8). The cause of these differences may be found in the increased size, activity, and water content of the older embryos. Any marked reduction of the water content, thus affecting the metabolic activity, would tend to depress the rate of oxygen consumption.

THE EFFECTS OF DEHYDRATION-REHYDRATION ON OXYGEN CONSUMPTION

The recovery of dehydrated prediapause and diapause eggs after rehydration has been noted earlier in this report (Table 3). The present experiment was conducted in an attempt to correlate the changes in susceptibility with the changes in the rate of oxygen consumption. Five stages of development (1-, 5-, 10-, 15-day, and diapause) and four concentrations (5, 10, 15, and 20 N) were used. The lengths of exposure were 5, 10, 15,



FIGS. 10–12.—Figure 10, changes in the rate of oxygen consumption of 1-day eggs immersed in hypertonic solutions for 5 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale. Figure 11, changes in the rate of oxygen consumption of 10-day eggs immersed in hypertonic solutions for 5 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale. Symbols as in Figure 10. Figure 12, changes in the rate of oxygen consumption of diapause eggs immersed in hypertonic solutions for 5 days and then transferred to N; arrow indicates time of transfer. Exposure time on reduced scale. Symbols as in Figure 10.

and 20 days. Following exposure to hypertonic solutions, the eggs were transferred to N. The responses of the dehydrated eggs to rehydration were dependent upon (1) the degree of dehydration, (2) the stage of development when dehydrated, and (3) the effect on diapause. Typical results have been chosen to illustrate these points.

The degree of dehydration was dependent upon the hypertonicity of the solution and the length of exposure (see Thompson and Bodine, 1936). There is, moreover, a corresponding depression in the rate of oxygen consumption (Figs. 4–8). If the rate of oxygen intake of eggs, while in a hypertonic solution, fell below 0.04 mm.^{3}/egg/hour (indicative of a great water loss) death of the eggs usually resulted. Such eggs, when transferred to N, consumed oxygen at a much higher rate (Fig. 9*C*, *D*). However, if the rate of oxygen consumption did not drop to such a low level, transfer to N was followed by an increase, later giving way to a decrease (Fig. 9*A*, *B*).

Rehydration of dehydrated eggs always elicited an increase in the rate of oxygen intake. In most instances this increase was followed by a decrease, the amount and rate of which were dependent upon the stage of development attained by the eggs prior to dehydration. If 1- and 5-day eggs were placed in hypertonic solutions and later transferred to N, then the decrease in the rate of oxygen consumption which followed the initial increase was quite marked (Figs. 10 and 11*A*, *B*, *D*). Such a decrease was slight or entirely absent if more advanced stages were used (Figs. 9*A*, *B* and 12*A*, *B*). In such cases the rate of oxygen intake eventually became constant. When the eggs were examined at the end of the experiment it was noted that most of the embryos had developed to the diapause condition.

The abbreviation or elimination of diapause by dehydration-rehydration noticeably affected the rate of oxygen consumption. Following rehydration there was a rapid intake of oxygen, the increase later becoming more gradual (Figs. 11*C* and 12*C*, *D*). Upon examination of the eggs at the end of the experiment, the developing postdiapause embryos responsible for the higher rates of oxygen consumption were noted.

DISCUSSION

The development of the embryos of *M. differentialis* is retarded when the eggs are dehydrated, the rate of growth being inversely related to the hypertonicity of the solution. Ludwig (1936) found that the duration of the pupal stage of *Popillia japonica* was increased by desiccation, this increase being inversely proportional to the water content of the pupae. The prolongation of development of grasshopper eggs gradually merges into a cessation of growth, this depending upon the embryonic stage and the concentration of the solution in which the eggs are immersed. Some embryonic growth is manifested in each of the hypertonic solutions (Table 1) although the percentage of embryos appearing in each concentration may differ (Fig. 1). Development in hypertonic solutions appears to be conditioned by the amount of water in the egg; cessation of growth occurs when the water content of the egg is lowered below a level sufficient to sustain further development. Bodine (1929) found that there was a corresponding increase in the water content of the egg as development progressed. From this it follows that a concentration which would dehydrate an egg rapidly would also effect a cessation of development more quickly. If the hypertonicity were less, development would continue at a retarded rate until there was an insufficient amount of water in the eggs to sustain further growth.

It is interesting to note that eggs of *Anisoplia austriaca* are able to extract water from 4 per cent NaCl, 2 per cent KNO_3, and 2 per cent $BaCl_2$ (Kerenski, 1930). It is not likely that eggs of *M. differentialis* are able to remove water from a hypertonic solution since a loss of turgidity has been noted following such treatment. Thompson and Bodine (1936) found that the postdiapause eggs of the grasshopper lose water to hypertonic solutions, the dehydration being rapid at first and then more gradual.

Recovery of dehydrated eggs, following rehydration, is dependent upon the physiological state of the egg when dehydrated and the concentration of the salt solution. The resistance of grasshopper eggs to dehydration has been determined, the order being (maximum to minimum): diapause, 15-, 10-, 1-, and 5-day eggs. Similar results have been found following irradiation (Evans, 1935, 1936). One of the probable factors concerned with the change in susceptibility is the activity of the cells. Diapause is a state of quiescence in which cellular activity is at a minimum. Slifer (1931) has shown that there is little or no mitotic activity in diapause embryos although such activity is extensive in prediapause ones. Slifer did not extend her investigation to postdiapause embryos but it is certain that such embryos would contain many dividing cells. Active differentiation is taking place with the formation of the embryo. It is during this period of active differentiation that the eggs are most susceptible to hypertonic solutions.

Ludwig (1936) noted that larvae of *P. japonica* could withstand a greater degree of dehydration when such water loss was not rapid. Although the eggs were not weighed in the present investigation, it is possible to compare the effects of different concentrations. In general the recovery is greater even after long exposures (20 days) to 5, 7.5, and 10 N than after short exposures (4 days) to 15 and 20 N.

Thompson and Bodine (1936) found that postdiapause eggs were usually killed after a water loss exceeding 2.5 mg. Such eggs, upon transfer to isotonic solution, became very swollen, the egg membranes frequently being ruptured. In the present investigation swelling and bursting have also been noted following rehydration of dehydrated eggs, the rupturing of the egg membranes occurring most frequently when 5-day eggs were so treated. Since the cuticle is secreted even when the eggs are in hypertonic solutions, this effect on 5-day eggs does not appear to be caused by the condition of the cuticular layers unless dehydration affects the thickness or some other characteristic of the white layer. It is not likely that the secretion of the white layer has progressed far in 5-day eggs. Secretion of this layer begins on the seventh day at 25° C. (Slifer, 1937).

In considering the effects of hypertonic solutions it is to be noted that eggs in various morphological stages behave differently. It is doubtful that these results are attributable entirely to the physiological state of the eggs. The effect of these solutions on the yolk is also to be considered since the yolk plays an important role in the environment of the embryo. The yolk/embryo ratio is extremely large in early prediapause, becoming very small in late postdiapause. Dehydration of the eggs might render the yolk cells incapable of liquefying the yolk and thus preventing its assimilation by the embryo. In such cases loss of water would tend to inhibit the development of the embryo.

The extent of development of postdiapause eggs in hypertonic solutions appears to be dependent upon the amount of water present. Adolph (1933) suggests that muscular contraction involves translocation of water. It is, therefore, quite possible that the degree of dehydration would tend to regulate the blastokinetic process. The findings, concerning the development of postdiapause eggs in hypertonic solutions, would serve to add credence to the above argument.

Previously only a reduction in the rate of oxygen consumption has been noted when eggs of *M. differentialis* were dehydrated (Bodine, 1933, 1934; Thompson and Bodine, 1936). The results of this investigation agree with the above findings where the data are comparable; however, an increase occurs in the oxygen intake of both 1-day eggs in all concentrations and of later postdiapause eggs in 2 and 3 N solutions. Bodine (1933) has suggested that the effects of hypertonic solutions are largely osmotic since NaCl solu-

tions, of the same osmotic pressure as the hypertonic balanced salt solutions, produced similar depressions in the rate of oxygen consumption.

Rehydration of dehydrated eggs is always followed by an increase in the rate of oxygen intake. The amount of oxygen consumed is dependent upon whether or not the effects of dehydration are reversible. Eggs which have been killed by dehydration usually possess a very high rate of oxygen consumption when rehydrated, this probably being caused by the actions of molds and bacteria. Irreversible results occur after the eggs have lost an excessive amount of water (see Thompson and Bodine, 1936). Depression of the rate of oxygen consumption by hypertonic solutions may be used as an index to irreversibility (Bodine, 1933). Any reduction of the water content of prediapause eggs, which lowers the rate of oxygen intake to 0.04 mm.^{3}/egg/hour, seems to be a sufficient water loss to prevent recovery.

SUMMARY

Retardation is noted when eggs are dehydrated, development continuing as long as the eggs contain a sufficient amount of water to sustain growth.

Five-day eggs are the most susceptible to hypertonic solutions, diapause the most resistant.

Dehydration-rehydration may serve to shorten or eliminate the developmental block.

The secretion of the yellow and white layers of the cuticle is not prevented by hypertonic solutions.

The rate of oxygen consumption of 1-day eggs, in hypertonic solutions, increases for 5 days before a decrease is noted. Dehydration of other prediapause stages results in an immediate decrease in the rate of oxygen intake.

The differences in the rates of oxygen consumption of eggs, in the various hypertonic solutions, are largely dependent upon the initial stage of the eggs.

Rehydration of dehydrated eggs results in an increase in the rate of oxygen intake, this increase usually being followed by a decrease.

LITERATURE CITED

ADOLPH, E. F. 1933. The metabolism and distribution of water in body and tissues. Physiol. Rev., **13**: 336–71.

———. 1936. Differential permeability to water and osmotic changes in the marine worm *Phascolosoma*. Jour. Cell. and Comp. Physiol., **9**:117–35.

BODINE, J. H. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., **2**:459–82.

———. 1933. The effects of hypertonic solutions on the oxygen consumption of a developing egg (Orthoptera). *Ibid.*, **6**:150–58.

———. 1934. To what extent is the developmental block dependent upon the metabolic activity of the embryonic cell? Proc. Nat. Acad. Sci., **20**: 640–44.

BOELL, E. J. 1935. Respiratory quotients during embryonic development (Orthoptera). Jour. Cell. and Comp. Physiol., **6**:369–85.

BOREI, H. 1935. Über die Einwirkung des Salzgehaltes auf den O_2-Verbrauch des Echinodermeneies. Z. f. Morph. u. Ökol. d. Tiere., **30**: 97–98.

BUXTON, P. A. 1932. Terrestrial insects and the humidity of the environment. Biol. Rev., **7**:275–320.

DIXON, M. 1934. Manometric methods as applied to the measurement of cell respiration and other processes. Cambridge, Eng.: University Press.

EVANS, T. C. 1935. Variations in susceptibility to x-rays of *Melanoplus differentialis* eggs during development. Physiol. Zoöl., **8**:521–29.

———. 1936. Qualitative and quantitative changes in radiosensitivity of grasshopper eggs during early development. *Ibid.*, **9**:443–54.

JAHN, T. L. 1935. Nature and permeability of grasshopper egg membranes. II. Chemical composition of membranes. Proc. Soc. Exp. Biol. Med., **33**:159–63,

JORDAN LLOYD, D. 1933. The movements of water in living organisms. Biol. Rev., **8**:463–81.

KERENSKI, J. 1930. Beobachtungen über die Entwicklung der Eier von *Anisoplia austriaca* Reitt. Z. f. angew. Ent., **16**: 178–88. (Cited by Buxton, 1932.)

KITCHING, J. A. 1934. The physiology of contractile vacuoles. I. Osmotic relations. Jour. Exper. Biol., **11**:364–81.

LUDWIG, D. 1936. The effect of desiccation on survival and metamorphosis of the Japanese beetle (*Popillia japonica* Newman). Physiol. Zoöl., **9**:27–42.

MELLANBY, K. 1935. The evaporation of water from insects. Biol. Rev., **10**:317–33.

SCHLIEPER, C. 1930. Die Osmoregulation wasserlebender Tiere. *Ibid.*, **5**:309–56.

———. 1935. Neuere Ergebnisse und Probleme aus dem Gebiet der Osmoregulation wasserlebender Tiere. *Ibid.*, **10**:334–60.

SLIFER, E. H. 1931. Insect development. II. Mitotic activity in the grasshopper embryo. Jour. Morph. and Physiol., **51**:613–18.

———. 1932*a*. Insect development. III. Blastokinesis in the living grasshopper egg. Biol. Zentralbl., **52**: 223–29.

———. 1932*b*. Insect development. IV. External morphology of grasshopper embryos of known age and with a known temperature history. J. Morph., **53**: 1–21.

———. 1934. Insect development. VI. The behavior of grasshopper embryos in anisotonic, balanced salt solutions. J. Exp. Zool., **67**: 137–57.

———. 1937. The origin and fate of the membranes surrounding the grasshopper egg: together with some experiments on the source of the hatching enzyme. Quart. J. Micr. Sci., **79**: 493–506.

SLIFER, E. H., and KING, R. L. 1934. Insect development. VII. Early stages in the development of, grasshopper eggs of known age and with a known temperature history. Jour. Morph. and Physiol., **56**:593–98.

THOMPSON, V., and BODINE, J. H. 1936. Oxygen consumption and the rate of dehydration of grasshopper eggs (Orthoptera). Physiol. Zoöl., **9**:455–70.

A STUDY OF THE MECHANISM OF CYANIDE INHIBITION: I. EFFECT OF CONCENTRATION ON THE EGG OF MELANOPLUS DIFFERENTIALIS[1]

(Seven figures)

W. A. ROBBIE, E. J. BOELL, AND J. H. BODINE
Zoölogical Laboratory, State University of Iowa

RESULTS obtained from studies of the depressing action of cyanide on the respiration of animal cells and tissues have been used by Warburg (1928) in support of a theory that respiration is due primarily to an activation of oxygen by an iron-containing enzyme. A complete discussion of this subject is found in Warburg's original works (1928) and in reviews by Dixon (1929) and Meldrum (1934) to which the reader is referred.

The present experiments have been designed primarily to determine the variations in cyanide sensitivity of the respiratory mechanism of grasshopper eggs using a manometric technique relatively free from difficulties ordinarily inherent in such work.

MATERIAL AND METHODS

Eggs of the common grasshopper, *Melanoplus differentialis*, with a known temperature history and of a known chronological and morphological age, were used throughout. Eggs studied during the prediapause and diapause periods of development were kept at 25° C. in the pods in which they were laid until they were desired for use. Those employed in the postdiapause determinations were kept in the pods at 25° C. until well into the diapause period at which time they were placed at 10° C. to break the diapause block. After two months or more at this temperature they were returned to 25° C. and resumed development as postdiapause eggs. Morphological ages of embryos were checked periodically.[2]

Measurements of respiration were made with Barcroft-Warburg manometers at 25° C. The number of eggs used in each determination varied from 35 to 200 depending upon the stage of development.

In cyanide experiments carried out by many of the earlier investigators the tissues or cells to be exposed were placed in a manometer flask with unneutralized KCN, using KOH as the CO_2 absorbing medium in the center well. Later Dixon and Elliott (1929) neutralized the cyanide to eliminate pH effects. With either of these techniques HCN gas distilled over from the suspension medium to the KOH in the center well, resulting in a rapid decrease of active HCN in the system. To avoid this difficulty some investigators replaced the KOH center well solution with one of KCN or NaCN sufficiently concentrated to absorb all of the CO_2 produced, and, because of its positive HCN ten-

[1] Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

[2] In this paper development is always considered as days at 25° C. since the morphological stages of development have been described by Slifer (1932) in such terms. For details concerning diapause and the physiology of blocked and developing eggs see Slifer (1932), Bodine (1932), Bodine and Boell (1934), and Boell (1935).

sion, to prevent any absorption of HCN from the suspension medium (Schmitt and Schmitt, 1931). Since this practice sometimes resulted in an increased HCN content of the fluid in which the cells or tissues were immersed, van Heyningen (1935), following Krebs (1935), employed as the center-well absorbing medium a mixture of KCN and KOH of such proportions that the HCN tension was equal to that of the immersion fluid.

To check the efficiency of these methods of exposure to cyanide for the present investigations, three sets of experiments were carried out, the results of which are shown graphically in Figure 1. In Experiments A and B one hundred 18-day prediapause eggs were run in each manometer with 3 cc. of 0.0025M KCN as the experimental fluid; the center-well solutions used are indicated in the explanation of the figure. At the end of a 3-hour

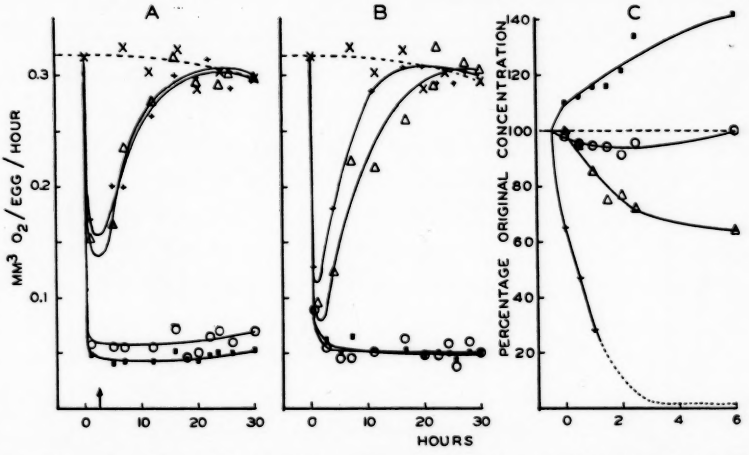


FIG. 1.—Comparison of techniques in the use of cyanide. (*A*) Arrow indicates the time at which the eggs were removed from the cyanide, washed, and returned to the manometers. (*B*) Eggs left in cyanide solutions continuously. (*C*) Variations in cyanide concentration in manometers containing same solutions as *A* and *B* but no eggs. Initial concentration of KCN = 0.0025M. × = control; eggs in water, 10 per cent KOH in inset. △ = unneutralized KCN, 10 per cent KOH in inset. + = KCN neutralized in pH. 7.0, 10 per cent KOH in inset. ■ = 0.0025M neutralized KCN as experimental fluid, 0.5M KCN in inset. ○ = 0.0025M neutralized KCN as experimental fluid, balanced KCN-KOH mixture in inset.

exposure to the KCN the manometer flasks in Experiment A were removed, the eggs washed, returned to the flasks with water as the suspension medium, and replaced in the bath. The flasks in Experiment B were not removed, the eggs remaining continuously in cyanide.

Chart C of Figure 1 shows the results of an analysis of cyanide concentration existing in the experimental fluid at various times after the start of the exposure. In the determination of each of the curves solutions similar to those used in A and B but containing no eggs were put in seven manometer flasks. These were then placed in the 25° C. water bath and shaken just as in Experiments A and B. At ½-hour intervals a manometer was removed and duplicate samples of the experimental fluid in the flask were analyzed by the method of Smith (1929).

An inspection of the curves shows that those eggs left continuously in cyanide solutions with KOH in the center well (Fig. 1*B*) recover about as quickly as those which are removed and washed at the end of a 3-hour exposure (Fig. 1*A*). This is apparently due to a diminished concentration resulting from the distillation of HCN to the KOH in the center well. With neutralized KCN less than 30 per cent of the cyanide originally present remains after the experiment has been run for an hour (Fig. 1*C*). The same graph shows that the cyanide concentration in the manometer flask containing strong unbalanced KCN center-well solution is 120 per cent of the original value at the end of 2 hours and 140 per cent within 6 hours. With the balanced KCN-KOH center-well solution recommended by Krebs and van Heyningen for 0.0025M KCN, the concentration at the end of the 6-hour period is 100 per cent of the original value, and during this time it does not vary more than 10 per cent (Fig. 1*C*).

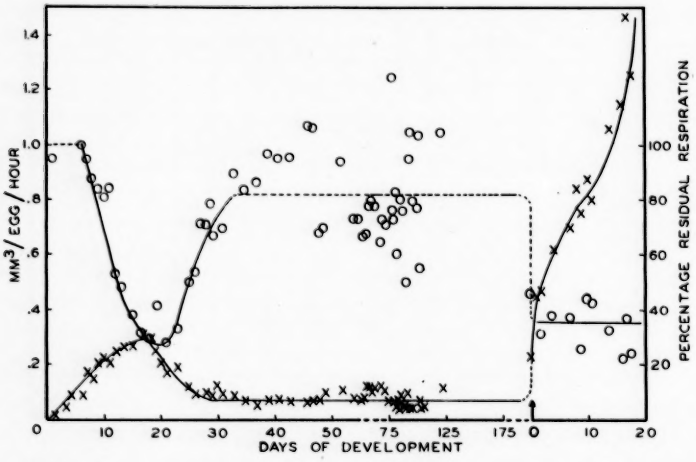


FIG. 2.—Variations in cyanide sensitivity during embryonic development. Left-hand ordinate represents O_2 consumed by control eggs (×). Right-hand ordinate shows percentage residual respiration of eggs treated for three hours with 0.001M KCN (○). Abscissa denotes days of development at 25° C. Prediapause period from 0 to 20 days; diapause from 21 days to arrow (diapause here terminated artificially as indicated in text); postdiapause period from 0 to 17–19 days (hatching). Each symbol represents an average value for the day indicated. Chart includes more than 250 experiments.

Difference in the recovery of eggs treated according to the technique of Schmitt and that of Krebs may indicate that the use of strong unbalanced KCN center-well solutions for exposures as short as 3 hours is an unsafe procedure.

The balanced center-well solutions recommended by Krebs (1935) have been used in all of the experiments described in this paper except in the preliminary ones indicated above. All of the work was carried out at a temperature of 25° C. and the exposures to cyanide, unless otherwise indicated, have been for periods of 3 hours.

PREDIAPAUSE PERIOD

Residual respiration of the egg in 0.001M KCN (Fig. 2) varies from 100 to 80 per cent of the control value during the sixth to the tenth day of development, but on the twen-

tieth day is only 30 per cent of the original level.[3] This increasing sensitivity to the action of cyanide as the egg grows older may be correlated with the increasing size of the embryo and the changes in the yolk cells during the prediapause period.

Following the twentieth day the cyanide sensitivity decreases to the diapause level. Figure 2 also indicates that the prediapause change in sensitivity is perhaps related to the course of normal O_2 uptake for the eggs. As the normal respiration of the egg increases with development the depressing effect of cyanide becomes more pronounced. Correspondingly, when the normal respiration decreases with the onset of diapause, the action of cyanide becomes less noticeable. This relationship of sensitivity and respiration

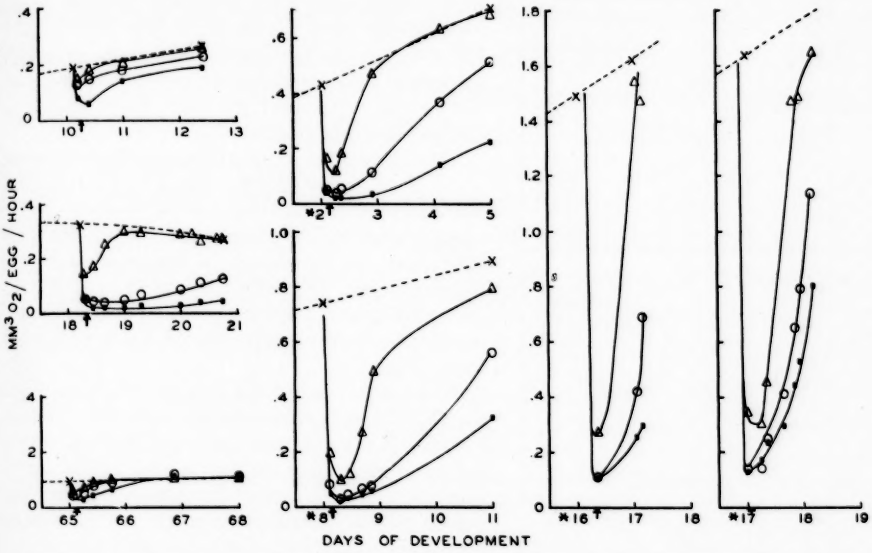


FIG. 3.—Exposure to and recovery from cyanide at different stages of development. Ordinates represent oxygen consumption in MM³ per egg per hour. Abscissae show the days of development. First numeral on abscissae shows age of eggs when cyanide was applied. Days marked with (*) indicate post-diapause age. × = control; △ = 0.0005M KCN; ○ = 0.0025M KCN; ■ = 0.01M KCN. Cyanide removed at arrow after 3-hour exposure.

level is shown also by Figure 5. Buchanan (1926) has similarly found that with *Planaria* a relatively greater depression is produced by cyanide as the rate of oxygen consumption increases.

Eighteen-day eggs are not only more depressed than those at the 10-day stage, but their recovery from exposure to cyanide is less rapid (Fig. 3). The former group treated for 3 hours at 25° C. with 0.01M KCN have recovered to 15 per cent of the original level of respiration at the end of 48 hours; the latter group have completely recovered by that time.

As one would expect, stronger concentrations of KCN depress respiration more than

[3] Whether there is a significant change in response after the formation of the chitinous cuticle on the sixth day (Slifer, 1937) is being investigated.

do weak ones. For the eighteenth day of prediapause development (Fig. 4*A*) the average residual respiration for 2 hours of a 3-hour exposure to concentrations of 0.01M–0.0005M KCN varies in a regular manner from 8.6 to 51.3 per cent of the control value.

DIAPAUSE PERIOD

As pointed out in the preceding section the sensitivity to cyanide is markedly lessened when the egg enters the diapause or block period and the normal respiration decreases to the diapause level. This is shown graphically in Figure 2. A comparison of *A* and *B* in Figure 4 shows that 0.01M KCN does not have as great a depressing effect on diapause eggs as 0.0005M KCN has on 18-day prediapause ones, although the former solution is twenty times as concentrated as the latter and the two stages are very similar morphologically.

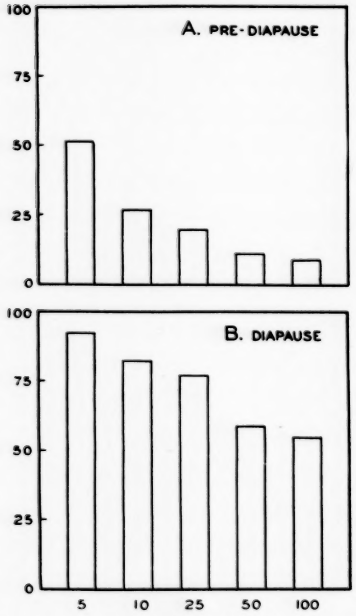


FIG. 4.—Effects of various concentrations of KCN on prediapause and diapause eggs. Exposures were for three hours at 25° C. with respiration measured over 2 hours of this period. Ordinates represent percentage residual respiration, abscissae show concentration of KCN × 10–4. Each column includes: in (*A*) average of 5 lots of 18-day prediapause eggs; in (*B*) 6 lots of 70–120-day diapause eggs.

The effect of concentration on the diapause egg is graphically seen in Figure 4*B*. Residual respiration levels for concentrations of KCN varying from 0.01M to 0.0005M range from 54.7 per cent to 92.1 per cent of the control value. Diapause eggs behave similarly to those at the 10-day prediapause stage by recovering completely from a 3-hour exposure to 0.01M KCN within 48 hours (Fig. 3).

Responses of different lots of diapause eggs to KCN show considerable variation. From Figure 2 it may be seen that the residual respiration of eggs exposed to 0.001M KCN varies from 28 to 124 per cent of the control value. Although eggs in diapause are all at the same morphological stage of development, their sensitivity to cyanide seems to vary with the normal level of respiration in a manner similar to that of prediapause eggs (Figs. 2 and 5). Those lots of eggs which respire at low levels are very resistant to the action of 0.001M KCN, while the ones with comparatively high respiratory rates may be depressed more than 80 per cent. The greatest sensitivity to cyanide during the period of developmental block occurs between the twentieth and twenty-fifth days. This may be due to the fact that the level of respiration during the onset of diapause is higher than that characteristic of the diapause period in general. The indications are that cyanide sensitivity during both the diapause and the prediapause periods may vary directly with the respiratory rate. The curves in Figure 5 should not be considered as being in any way absolute boundaries between a cyanide-sensitive and a cyanide-insensitive portion

of the respiration of the egg, as stronger concentrations of KCN produce further depression of the respiration in a still readily reversible fashion.

This increased cyanide sensitivity of eggs with a high respiratory rate as compared with those with a low rate might indicate that the potency of the diapause block is not as great in the former type of eggs as in the latter (Bodine and Boell, 1937). The increased respiration seems not to be associated with any visible morphological changes of the embryo, but may represent some type of physicochemical change similar to that which Runnström (1930) has suggested occurs in the sea urchin egg during transition from the unfertilized to the fertilized state.

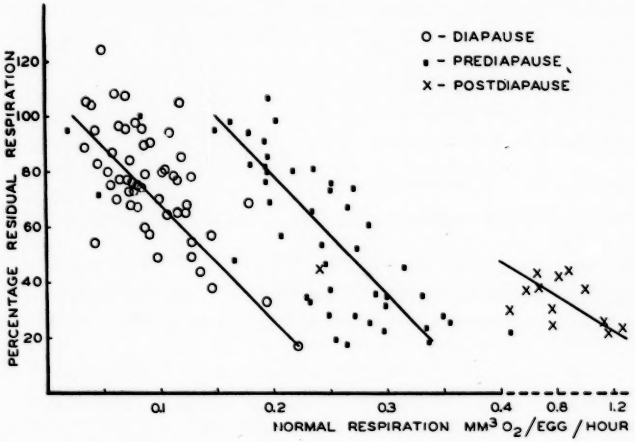


FIG. 5.—Curves showing variation in sensitivity to cyanide with variation in magnitude of respiratory rate. Ordinate represents percentage residual respiration; abscissa, respiratory rate. All eggs exposed for 3 hours at 25° C. to 0.001M KCN.

The similarity of response of 18-day prediapause and postdiapause eggs, and 10-day prediapause and diapause eggs (Figs. 2, 3, and 6) might suggest that there are two types of respiratory mechanisms active during embryonic development, one of which accounts for most of the respiration of the former group, the other for that of the latter. The stages between the tenth and eighteenth prediapause days and those diapause lots which have a higher respiration and are more sentitive to KCN than the normal may represent a combination of these two mechanisms. An investigation of this phase of the problem is being carried out at the present time.

POSTDIAPAUSE PERIOD

After the diapause block has been destroyed by exposure to low temperature and the eggs are again placed at 25° C., development is resumed and the respiratory rate rises rapidly. This is indicated graphically by the lower curve in Figure 2. The course of this curve representing normal egg respiration confirms the work of Bodine (1929) and Boell (1935).

It has been previously shown by Bodine (1934) that a marked susceptibility to KCN follows the termination of the diapause period. Confirmation of this is seen in the effect of 0.001M KCN on eggs treated within 24 hours of removal from the low temperature. Eggs so treated show a greater depression of respiration than any of those diapause eggs exposed to cyanide after the twenty-fifth day from laying (Fig. 2).

From the results of experiments graphically represented in Figures 2 and 6 it may be concluded that no significant change in sensitivity takes place during the postdiapause

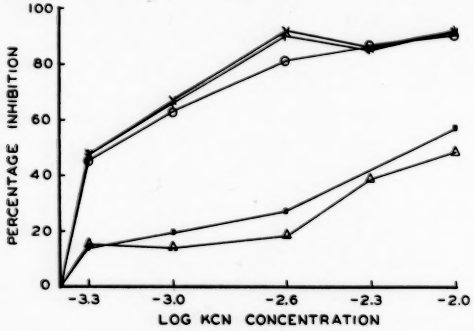


FIG. 6.—Effect of concentration of KCN. Points represent averages of experiments run with many different lots of eggs. All exposures were for three hours at 25° C. ■ = 10-day prediapause eggs; ○ = 18-day prediapause; △ = diapause; + = 0–9-day postdiapause age; × = 9–18 day postdiapause.

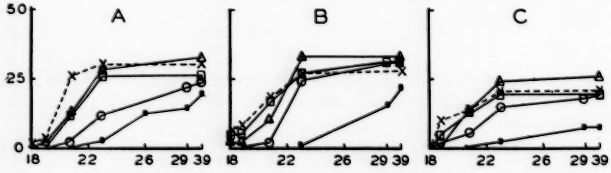


FIG. 7.—The effect of cyanide on time of hatching of eggs treated on zero postdiapause day (*A*), seventh day (*B*), and seventeenth day (*C*). × = control; △ = 0.0005M KCN □ = 0.001M KCN; ○ = 0.005M KCN; ■ = 0.01M KCN. All exposures for 3 hours at 25° C. Ordinates represent number hatching; abscissae, postdiapause age.

period. The level of residual respiration as percentage of the control value in the various concentrations of cyanide employed is about the same throughout the period as at the eighteenth prediapause day.

Recovery of respiration after exposure of the eggs to cyanide proceeds more rapidly during the latter part of the postdiapause period than at the start (Fig. 3), but the delay in hatching caused by exposure is similar for eggs treated on the zero day of postdiapause development and those treated on the sixteenth day (Fig. 7). Those treated with 0.01M KCN hatch about a week later than the controls. The number of embryos which hatch is apparently not a good indication of the effect of cyanide on development; the embryos of those eggs treated after the middle of the postdiapause period, although delayed,

progress in most cases to the morphological state typical of the eighteenth postdiapause day, but they seem to be unable then to escape from their shells. The fact that as many as 40 per cent of the postdiapause eggs treated for 3 hours at 25° C. with 0.01M KCN do hatch, and are able to live after hatching, shows that this concentration is at least partially reversible under these conditions.

SUMMARY

1. A study has been made of the effect of cyanide on the respiration of the egg of the grasshopper, *M. differentialis*, throughout its embryonic development, using the technique developed by Krebs and van Heyningen.

2. Methods of exposure to cyanide and their suitability for physiological uses are discussed.

3. An increasing sensitivity of eggs to cyanide is noted from the time of laying until the onset of diapause. Sensitivity to 0.001M KCN then decreases rapidly to a diapause level about 55 per cent less than the prediapause maximum.

4. During both the prediapause and the diapause periods the sensitivity seems to vary directly with the normal respiratory level of the eggs.

5. After diapause has been terminated by exposure to low temperature the sensitivity rises to about the same level as the prediapause maximum, and remains constant at this level.

6. Concentrations of KCN varying from 0.0005M to 0.01M show a progressively powerful action in depressing respiration. At the eighteenth prediapause day the inhibition produced by this range of KCN concentrations varies from 45 to 90 per cent of the control value. For the postdiapause period the range is from 47 to 92 per cent.

7. Ten-day prediapause and diapause eggs behave similarly toward cyanide, the inhibition varying from 14 to 57 per cent over the concentration range 0.0005—0.01M.

Curves showing recovery after exposure to KCN are given for prediapause, diapause, and postdiapause eggs.

9. A 3-hour exposure to KCN delays the time of hatching from a day to a week depending upon the concentration employed and regardless of the postdiapause age at the time of the application. This is true even if the exposure is made on the day immediately preceding that on which hatching normally occurs.

10. Three-hour exposures to 0.01M and 0.005M KCN result in a lowered percentage hatch, but the fact that 40 per cent or more of the control value of eggs hatch when treated with the former concentration shows that for this duration of exposure the dose is at least partially reversible.

LITERATURE CITED

BODINE, J. H. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., **2**:459.

———. 1932. Hibernation and diapause in certain Orthoptera. II. Response to temperature during hibernation and diapause. Physiol. Zoöl., **5**:538.

———. 1934. The effect of cyanide on the oxygen consumption of normal and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., **4**:397.

BODINE, J. H., and BOELL, E. J. 1934. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). Jour. Cell. and Comp. Physiol., **5**:97.

———. 1937. The action of certain stimulating and inhibiting substances on the respiration of active and blocked eggs and isolated embryos. Physiol. Zoöl., **10**:245.

BOELL, E. J. 1935. Respiratory quotients during embryonic development (Orthoptera). Jour. Cell. and Comp. Physiol., **6**:369.

BUCHANAN, J. W. 1926. Depression of oxidative metabolism and recovery from dilute KCN. Jour. Exper. Zoöl., **44**:285.

DIXON, M. 1929. Oxidative mechanisms in animal tissues. Biol. Reviews, **4**:352.

DIXON, M., and ELLIOTT, K. A. C. 1929. The effect of cyanide on the respiration of animal tissues. Biochem. Jour., **23**:812.

HEYNINGEN, W. E. VAN. 1935. The inhibition of respiration by cyanide. Biochem. Jour., **29**:2036.

KREBS, H. A. 1935. Metabolism of amino acids. III. Deamination of amino acids. Biochem. Jour., **29**:1620.

MELDRUM, N. U. 1934. Cellular respiration. London: Methuen.

RUNNSTRÖM, J. 1930. Atmungsmechanismus und Entwicklungserregung bei dem Seeigelei. Protoplasma, **10**:106.

SCHMITT, F. O., and SCHMITT, O. H. A. 1931. The nature of the nerve impulse. II. The effect of cyanide upon medullated nerves. Amer. Jour. Physiol., **97**:302.

SLIFER, E. H. 1932. Insect development. IV. External morphology of grasshopper embryos of known age and with a known temperature history. Jour. Morph., **53**:1.

———. 1937. The origin and fate of the membranes surrounding the grasshopper egg; together with some experiments on the source of the hatching enzyme. Quart. Jour. Mic. Soc., **79**:493.

SMITH, R. G. 1929. A method for the quantitative determination of cyanide in small amounts. Jour. Amer. Chem. Soc., **51**:1171 (Part I).

WARBURG, O. 1928. Über die katalitischen Wirkungen der lebendigen Substanz. Berlin: Julius Springer.

COMPARISON OF QUANTITATIVE PRECIPITIN TECHNIQUES AS INFLUENCED BY INJECTION PROCEDURES

(Seven figures)

HAROLD R. WOLFE AND JOSEPH G. BAIER, JR.
Department of Zoölogy, University of Wisconsin

THE specificity of the precipitin reaction has been found to be altered by continued injections of antigen. Magnus demonstrated this in 1908 using plant tissue extracts. Lake, Osborne, and Wells in 1914 showed that, when purified vegetable proteins were used as antigens, the specificity of the resulting immunological reactions, as measured by precipitin, anaphylactic, or complement fixation tests, was altered by continued injections. Satoh (1933) distinguished between *präzipitingehalt* and *präzipitintiter* and found the number of injections affected the former considerably. Antisera produced by the larger number of injections were less specific and were not so easily affected by higher dilutions as were the antisera resulting from fewer injections. Hooker and Boyd (1934) using crystalline albumen of duck and hen eggs found that earlier bleedings exhibit more specificity than later bleedings.

Quantitative studies of the effects of injection procedures on the serum precipitin reaction have been reported by Wolfe (1935, 1936). In these experiments he used rabbits as antibody builders and the serum proteins of various mammalian bloods as antigens. When small quantities (2–3 cc.) of undiluted serum proteins were injected in three doses into rabbits, the resulting antisera were more specific than those produced by injections of 10 cc. or more in five to ten injections. Antisera produced by the former method usually yielded higher titered reactions but seldom gave "extra-group" response. Reinjections of the animals with 1.5 cc. of undiluted homologous antigen at intervals of a month or more resulted in the production of aspecific precipitins, but reinjections with minute quantities restored the antibody content of the blood without affecting the quality of the precipitins. These data were believed to be of especial importance to the serologist who is interested in studies of animal or plant relationships. Greater uniformity of results could be expected when injection procedures were controlled and the value of the precipitin test in studies on phylogeny would be enhanced.

If the antisera were produced by injections of the smaller quantity of antigen or by reinjections of minute quantities, the ring-test titers were found to be much higher than the flocculation titers after 1 hour of incubation at 37.5° C. ± 1. It was also noted that the amount of the precipitate of an antigen-antibody mixture, as determined macroscopically, varied with the amount and number of injections. Increased quantities of antigen injected into a rabbit and additional series of reinjections of undiluted antigen resulted in greater amounts of precipitate in the tests performed in vitro. There was no noticeable correlation between "ring-test" titer and precipitate formation. The interfacial titer reached a maximum after one or two injection series, but the precipitating power seemed to increase with each reinjection of undiluted antigen.

There are various quantitative methods for studying the precipitin reaction. The more accurate ones employ measurements of the precipitate by (1) direct volumetric deter-

mination, (2) dry-weight measurement, and (3) nitrogen analysis. The greater reliability of one technique over the other has not been established to the satisfaction of all workers. All these techniques are different expressions of the amount of precipitate formed, and this, it is assumed, is an index of the quantity of antibody present in an antiserum. It is known that the precipitate consists of antigen and antibody. Therefore, in an antigen-antibody system, at points of optimal proportions where the supernatant fluid contains practically no antigen or antibody, if the antigen is constant and the amount of precipitate varies, it is an indication of the variance in the amount of antibody.

Theoretical considerations of the precipitin system have been made by Heidelberger and his co-workers. They have employed various simple antigens and have concluded that the reaction follows the law of mass action. Heidelberger, Kendall, and Soo Hoo (1933) reported the effects of successive courses of injections on the precipitin content of the rabbit's blood. They found two types of response. In one type of response the rabbit's blood reached a relatively low but maximum content with one course of injection, and a second type of response occurred in which the precipitin content increased with successive courses to a maximum. Their data almost entirely substantiated their first point. Taylor, Adair, and Adair (1934) and Heidelberger and Kendall (1935) reported effects of more than one course of injections. The latter authors concluded from their results and the results of Taylor, Adair, and Adair that "the sera exhibited progressive changes. . . . . These changes are believed to consist in the formation of more and more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule." These results can be readily understood and are not unexpected, for the rabbits were injected for long periods of time with a comparatively large amount (total) of antigen. The greatest possible antibody response should be expected with such treatment. The studies did not include the earlier stages of precipitin formation in the rabbit.

Baier (1933) has been able to show graphically the relations of antigen and antibody in the precipitin reaction and in what respect various chemical and physical agents may modify their interaction. The technique employed in this study was reported by Boyden and Baier (1929) and is concerned with volumetrically measuring the amount of precipitate formed in the reaction between known amounts of an antigen with its specific antiserum by means of Van Allen thrombocytocrits. They have shown their quantitative volumetric technique to be fairly simple, rapid, and statistically reliable.

The purposes of the research work described in this report are to show, by the use of a volumetric quantitative technique of known accuracy, (1) the effects of injection procedures on the precipitating power of antiserum from the initial appearance of antibody through successive courses of injections, (2) a comparison of the volumetric precipitate formation and ring-test titer, and (3) a correlation of these results with previous work.

MATERIALS AND METHODS

Sterile buffered saline solutions of egg albumen, beef oxyhemoglobin, and the albumen and pseudoglobulin fractions of pig and dog sera were used as antigens and injected into healthy rabbits. The initial series of injections consisted of three doses given on alternate days; the first dose consisting of 0.5 cc. and the total amount 3 cc. If the titer ("ring" test) was then sufficiently high, the animals were bled 12 days after the last injection. Then, at intervals of a month or longer, the animals were given repeated series of rein-

jections, each series consisting of 1.5 cc. of undiluted homologous antigen administered in two doses on alternate days. Ten days after the last series of reinjections, the animals were bled. In several cases the animals were also bled just previous to each reinjection series.

The protein content of the solutions used was determined by the modified Macro-Kjeldahl method and was based on the total nitrogen content. The antigens varied from 0.94 to 5.11 gm. protein per 100 cc of solution, and this was considered the undiluted antigen. Two per cent standard solutions (1:50 dilution of the undiluted antigen with buffered saline) were made, and for ring-test titers and volumetric determinations these solutions were used in serial dilutions. The crystalline egg albumen was prepared according to the method of Hopkins and Pinkus (1898), and the oxyhemoglobin after the method of Marshall and Welker (1913). The individual proteins of the blood serum were fractionated by the procedures described by Hektoen and Cole (1927).

Each rabbit was given a definite number, and the initial antiserum obtained was numbered similarly. The letter "A" was added to this number to indicate that the blood was taken from the rabbit after a rest period of 30 or more days and the number "1" added if the animal had been reinjected 10 days previously. Thus antiserum ♂152-A1 was from a male rabbit bled for the third time and taken 10 days after the second injection series. The sera were stored in ampules after sterile filtration and kept at lowered temperatures.

Reactions were determined only for homologous antigens. The term "titer" when used in this paper refers to the ring-test titer, while the term "precipitating power of an antiserum" refers to the production of a volumetric measurable amount of precipitate in an antigen-antibody mixture. To understand the volumetric data as presented in this paper, suppose we interpret the curves from one set of volumetric determinations, such as in Figure 3. Beginning at the junction of the abscissa and the ordinate and progressing toward the direction of greater antigen dilution (lesser antigen concentration) we can see that the volume of precipitate rapidly reaches a maximum and then gradually decreases. The part of the curve to the left of the maximum represents the "zone of inhibition," where the antigen, being in excess, has caused a re-solution of the precipitate. The part of the curve to the right represents the volume of precipitate obtained when the antigen is relatively deficient. The peak of the curve is relatively at a concentration of antigen and antibody where neither one is in excess. Therefore, a shift in this peak to the left, where the antigen concentration becomes greater, shows an increase in "precipitating antibody." Also, an increase in the amount of precipitate produced shows an increase in "precipitating antibody," so that either one, or both, of these factors may be employed in describing the potency of an antiserum in its precipitating power (Baier, 1933, Fig. 6).

RESULTS

Table 1 records data on antisera produced by seven rabbits, and exemplifies the method of numbering of the antisera. Five other rabbits giving similar results are not listed. The dates of injections and of bleedings should be correlated with the interfacial test titer and with the volumetric method of measuring the precipitate. While antisera of very high titer did not necessarily yield a measurable precipitate, precipitating sera always were of high titer. One injection series often resulted in a maximum titer, but at times two series were necessary (especially against hemoglobin) for its production. Further reinjections did not raise the titer but usually resulted in an increased amount of precipitate as determined by measurements with the thrombocytocrits. It may also be

noted that, if a rabbit is not reinjected for a long period of time (a month or more), its serum will show a loss of "precipitating power," but the "titer" will not have decreased as greatly.

TABLE 1

EXPERIMENTAL DATA—ANTIGENS USED AND "PROPERTIES" OF ANTISERA

Antiserum Number	Antigen Injected and Protein Content	Date of Injection*	Date of Bleeding	Titer	Measurable Precipitate Formation	Refer to Fig. No.
♂155	Beef oxyhemoglobin	1-14-35	1-30-35	0	−	.....
-I	5.11 gm. per 100 cc.	2-23-35	3-6-35	12,800	−	.....
-II		4-1-35	4-12-35	25,600	+	1
-IIA		.....	7-1-35	6,400	−	.....
-IIA1		7-11-35†	7-23-35	12,800	−	.....
-IIA1A		.....	10-9-35	6,400	−	.....
-IIA1A1		11-13-35	11-24-35	25,600	+	1
♂170		3-11-35	3-25-35	400	−	.....
-I		4-15-35	4-27-35	25,600	−	.....
-II		5-20-35	6-1-35	51,200	−	.....
-III		8-21-35	9-2-35	51,200	+	2
-IIII		11-25-35	12-7-35	51,200	+	2
-IIIIA		.....	2-17-36	12,800	−	.....
-IIIIA1		2-29-36	3-12-36	51,200	+	2
♂152	Crystalline egg albu-	1-14-35	1-30-35	25,600	−	.....
-I	men, 4.05 gm. per	2-23-35	3-6-35	51,200	+	3
-II	100 cc.	4-1-35	4-12-35	51,200	+	3
-IIA		.....	8-1-35	25,600	−	.....
-IIA1		7-11-35†	7-23-35	25,600	−	.....
♀167		3-4-35	3-20-35	51,200	−	.....
-I		4-16-35	4-28-35	51,200	+	4
-II		5-20-35	6-1-35	51,200	+	4
♀163	Albumen fraction of	3-4-35	3-20-35	25,600	+	5*a*
-I	dog serum, 3.55 gm.	4-15-35	4-27-35	25,600	+	5*a*
-II	per 100 cc.	5-22-35	6-3-35	25,600	+	5*a*
-IIA		.....	8-22-35	25,600	−	.....
-IIA1		9-4-35	9-16-35	25,600	+	5*b*
-IIA11		12-2-35	12-14-35	25,600	+	5*b*
♀173		4-1-35	4-15-35	800	−	.....
-I		5-6-35	5-18-35	25,600	−	.....
-II		6-17-35	6-29-35	51,200	+	6
-III		7-25-35	8-6-35	51,200	+	6
-IIIA		.....	10-21-35	12,800	−	.....
♂156	Pseudoglobulin frac-	3-11-35	3-25-35	1,600	−	.....
-I	tion of dog serum	4-16-35	4-28-35	6,400	+	7
-II	0.94 gm. per 100 cc.	5-20-35	6-1-35	25,600	+	7
-IIA		.....	8-24-35	800	−	.....

* First injection of series.

† 2 per cent solution injected instead of undiluted antigen.

Antibeef oxyhemoglobin sera from two rabbits yielded very similar results. It was necessary to inject rabbits ♂155 and ♂170 with two series of antigens before they re-

sponded with high-titered antisera. Further reinjections with undiluted antigen did not increase this value (within the range of error of the test which is one tube). A measurable precipitate was obtained after three injections into rabbit ♂155 and after four series in rabbit ♂170. Figure 1 shows that further treatments with undiluted antigen increased the quantity of antibody in the antigen-antibody mixture as indicated by a shift of the curve to the left. Figure 2 demonstrates the increased antibody content of series ♂170 resulting from successive courses of injections, for there is a larger quantity of precipitate formed as well as a shift of the peak of the curve to the left. Antiserum ♂155-11A1 (Table 1) was produced by reinjections with a 2 per cent solution, but it did not have any "precipitating power." The results obtained with antisera ♂155-11A, ♂155-11A1A, and ♂170-1111A (Table 1) illustrate the effects that a long period of rest, occurring after injections, have on "titer" and "precipitating power." The type of curve resulting when hemoglobin was used as antigen was different from the curves obtained by the use of other antigens. Analyses of these differences have not been made.

Figures 3 and 4 are the precipitate curves for two series of antisera against crystalline egg albumen. The initial series of injections in each rabbit produced high-titered antisera without producing measurable amounts of precipitate. Reinjections of the two rabbits about a month later with 1.5 cc. of undiluted antigen resulted in antisera that yielded much precipitate, but whose titers did not change outside the limits of error of the test.

Serum ♂152-2 gave a much greater precipitate than did ♂152-1. In order to obtain accurate measurements with ♂152-2, it was necessary to dilute one volume of it with one volume of buffered saline (resulting dilution 1:2), for without dilution the thrombocytocrits that were used at the time were not large enough to measure the precipitate obtained from a mixture of antibody and antigen at the point of highest precipitate formation. The effect of antiserum dilution as seen in the comparison of graphs ♀163 undiluted and ♀163 diluted 1:2 (Fig. 5*a*) is to lower proportionately the amount of precipitate obtained volumetrically and to shift the peak to the right without otherwise altering the characteristics of the curve. The peak of the curve for ♂152-2 is also much to the left of the peak for ♂152-1, even though the former was diluted 1:2. This is similar to the results shown in Figures 1 and 2. When the rabbit was bled about 3 months after the ♂152-2 bleeding, its blood was still of high titer but lacked precipitating power, which was not restored with reinjections of 1.5 cc. of a 2 per cent antigen solution.

The volume of precipitate of ♀167-2 and its homologous antigen was considerably greater than that of ♀167-1 when used undiluted. The former yielded measurable quantities of precipitate when diluted with three parts (1:4 dilution) of buffered saline, and without dilution the volume of precipitate obtained was so great as to be immeasurable.

Two rabbits were injected with the albumen fraction of dog serum. The initial set of injections into rabbit ♀163 resulted in a titer of 1:25,600, and four series of reinjections with undiluted antigen had no effect on this value. Serum ♀163 was the only one that yielded a precipitate (Figs. 5*a* and 5*b*) following one series of injections. The first and second reinjection series increased the amount so greatly that the antisera had to be diluted in order to allow for volumetric determinations. On the other hand, the third and fourth series of reinjections did not increase the quantity of precipitate. Usually the measurements of precipitate were made within 2 or 3 months after the antiserum was secured, but in these cases the determinations were made after 18 months had elapsed. At the present time it is not known whether or not this result indicates that the precipitating

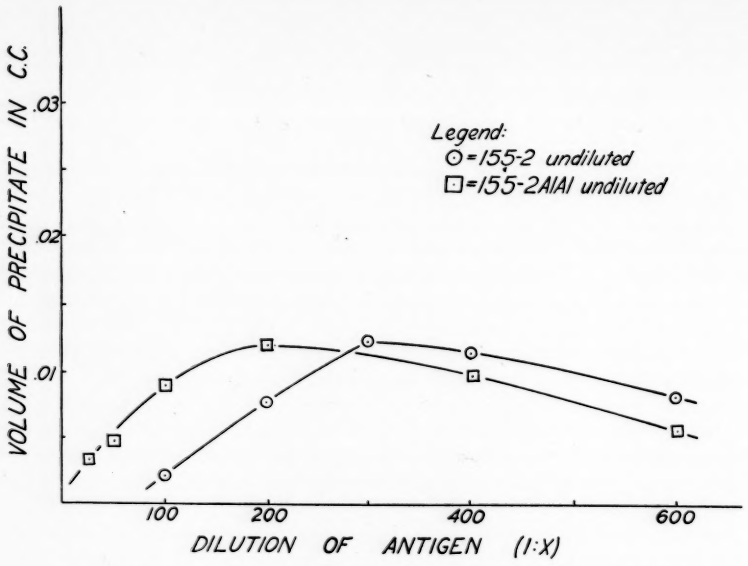


FIG. 1.—Antiserum of rabbit ♂155 against antigen BH-100

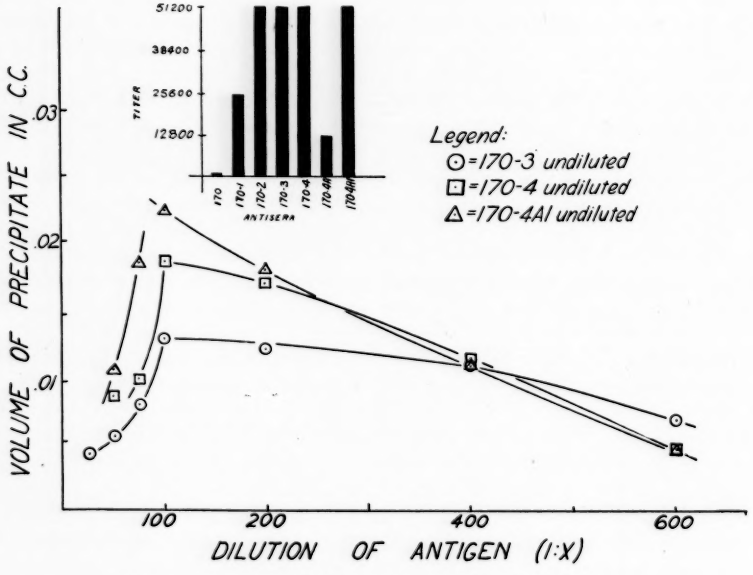


FIG. 2.—Antiserum of rabbit ♂170 against antigen BH-100

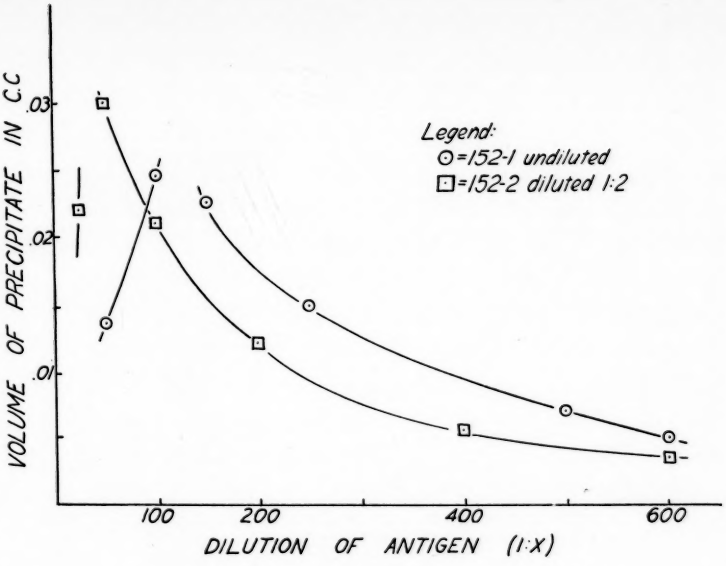


FIG. 3.--Antiserum of rabbit ♂152 against antigen CE-21

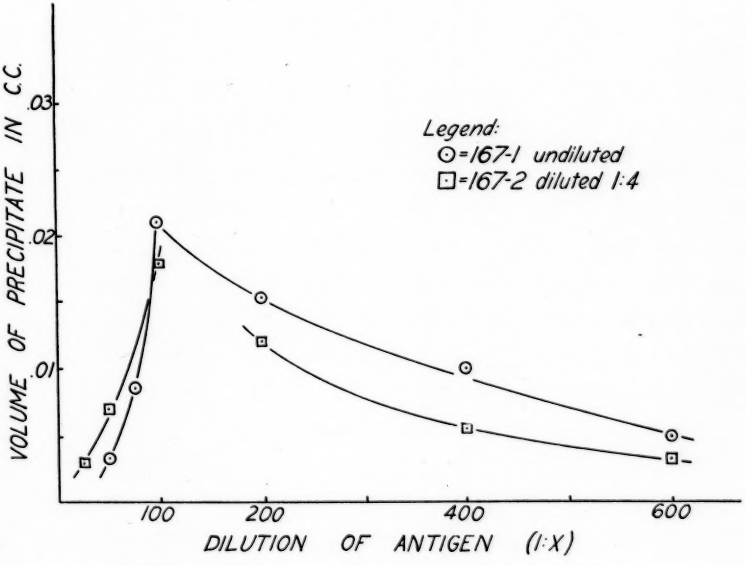


FIG. 4.—Antiserum of rabbit ♀167 against antigen CE-21

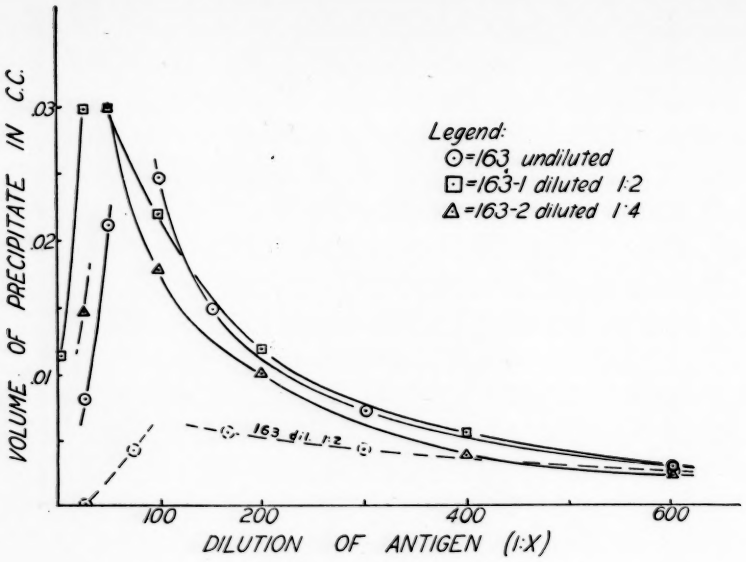


FIG. 5*a*.—Antiserum of rabbit ♀ 163 against antigen DA-1

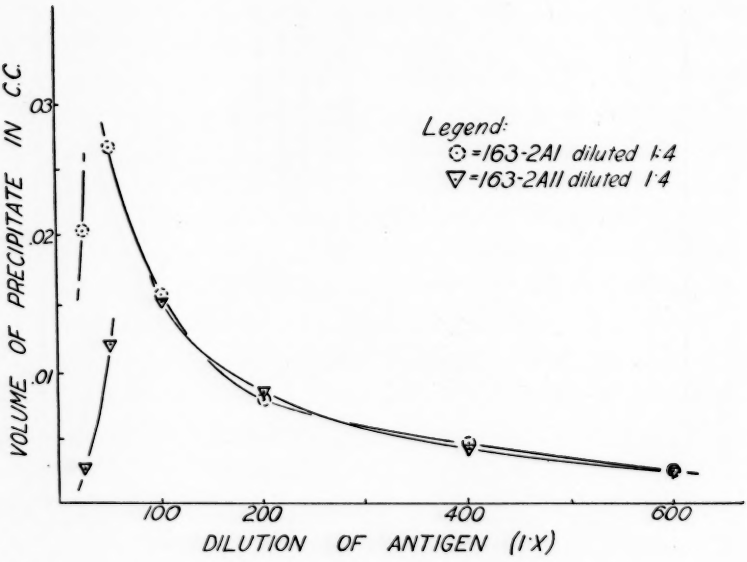


FIG. 5*b*.—Antiserum of rabbit ♀ 163 against antigen DA-1

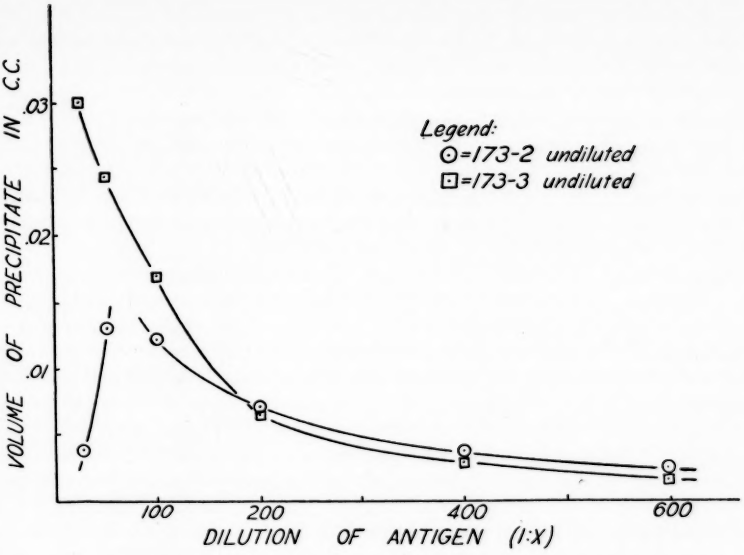


FIG. 6.—Antiserum of rabbit ♀ 173 against antigen DA-1

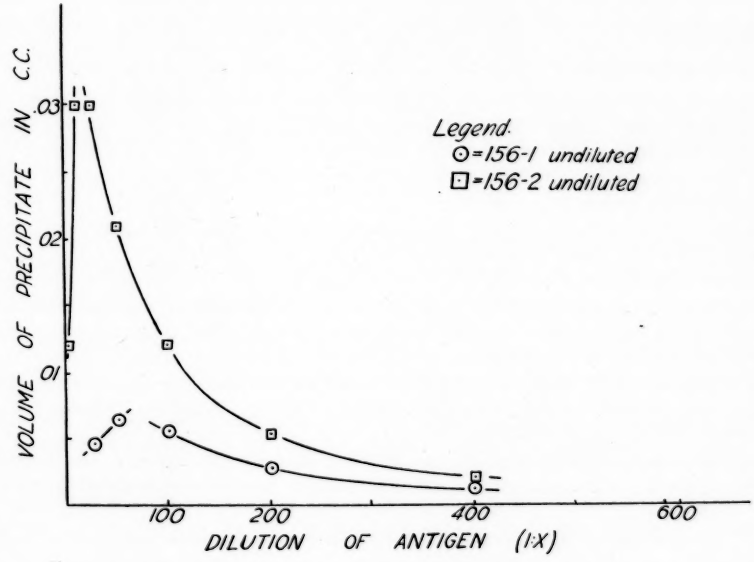


FIG. 7.—Antiserum of rabbit ♂156 against antigen DP-1

power of a serum reaches a maximum and cannot be raised above this point, or whether other factors, such as age of the antiserum, may affect the results. These factors are being studied in further experiments.

Figure 6 records the precipitating curves for two antisera against the albumen fraction of dog serum, and Figure 7 represents curves for antisera against the pseudoglobulin fraction. Again, the effects of reinjection with undiluted antigen have been shown to cause an increase in the precipitating power of an antiserum when reacted against its homologous antigen, while without reinjection, for a month or longer, this power of the rabbit's serum to form a precipitate decreases (Table 1).

DISCUSSION

The experiments have been carried out in two steps. One of the authors injected the animals and made the ring-test titers, while the other, not knowing the procedures or results of this, made the volumetric determinations. The tabulation and correlation of the results showed a noteworthy consistency of the data. Certain facts that have not been fully dealt with by other workers were demonstrated in regard to the effect of injection methods on the properties of precipitins.

It can be observed from a study of any one of the figures that the first precipitating antiserum obtained from a rabbit has the highest peak of the curve, showing maximum precipitate formation at a point where the antigen dilution is of a relatively low concentration, while the same rabbit serum obtained after a second and third series of injections shows its point of maximum precipitate formation at a lower dilution (higher concentration of antigen). In attempting to explain this, let us recall that the "zone of inhibition" is that portion of the curve where the antigen is in excess, and which therefore causes re-solution of the precipitate. As a result, the antisera of the second and succeeding injection series must contain a greater quantity of precipitating antibody, for in many graphs the "zone of inhibition," in spite of using diluted antisera, continues to be moved toward the left of the graph. That there is no exact correlation between the increase in precipitating power of an antiserum and its "titer" is readily shown from this analysis of the curves. This is suggestive that there are two kinds of antibodies produced —the "titer"-producing antibody and the "precipitate"-forming antibody. A study of this is now being made.

It is an interesting fact that, with the methods and materials used, the ring-test titers early reached a maximum and could not be raised while the volume of precipitate increased with injections of undiluted antigen. Is it possible that the titer could not be raised because the amount of protein in the higher dilutions was infinitesimally small? The facts clearly demonstrate the differences between the "titer" of an antiserum and its ability to precipitate in the presence of varying amounts of an antigen. A very similar variation is reported by Wolfe (1935, 1936), where an antiserum may at one time have a high titer and be very specific toward its antigen but on further injection of the same rabbit producing this antiserum still have the same high titer and yet be very aspecific.

Apparently these methods of study are showing us more conclusively that antisera to be comparable must be produced following definite procedures depending upon the "type" of antiserum desired. To the zoölogist who is studying animal relationships the ring-test method seems most applicable. If such factors as injection procedures and protein content of antigen, which was emphasized by Boyden (1926), are taken into consideration, the value of the precipitin test in such studies would be greatly increased.

The medicolegal worker would also benefit were he to consider these factors. Antisera that yield measurable amounts of precipitate give valuable data in interpreting the mechanism of the reaction, but whether this type of antiserum can show, as accurately as the "ring" test, phylogenetic relationships has not been shown. It would be of interest and significance to know how the variance in quantity of antibody in antisera would affect the cross-reactions when the antisera give measurable precipitates.

Six monkeys, the weights of which were approximately the same as those of the rabbits, were injected and reinjected with undiluted horse serum. The quantities injected were the same as those used in rabbits, but the protein content of the serum was slightly higher. The monkeys received from five to eight series of injections. The responses were very different from those obtained in the rabbits. The titers were weak, with a maximum of 1:12,800, but more usually of 1:3,200. There was very little precipitate formation, and the antiserum was very specific in the few cross-tests that were made. A comparison of the monkey and rabbit, of course, demonstrates that, when horse-serum proteins are used and injection methods are similar, the formation of precipitins is very different in the two species. This is a species difference as far as we can determine, unless factors that we haven't anticipated have been overlooked. What is the cause of this difference in response? We find no answer but use the oft-repeated term "species individuality."

SUMMARY

1. Rabbits have been injected with mammalian blood proteins and crystalline egg albumen.

2. High-titered antisera were produced by one or two series of injections of undiluted antigen, and this titer could not be raised by further injections.

3. Continued reinjections, as far as the experiments went, resulted in increased precipitate formation and a shift of the curve toward the region of greater antigen concentration showing an increase in the antibody causing precipitation.

4. Two injection series were usually necessary to produce antisera that had precipitating power; using hemoglobin solutions, three and four injection series were needed.

5. The precipitating curves resulting with hemoglobin as antigen differed from the type of curves with the other antigens.

6. Indications are that at least two types of antibodies are present: (1) a "titer"-producing antibody and (2) a "precipitate"-forming antibody.

7. The zoölogical significance of the data is emphasized.

LITERATURE CITED

BAIER, J. G., JR. 1933. Quantitative studies on precipitins. Physiol. Zoöl., **6**:91.

BOYDEN, A. A. 1926. The precipitin reaction in the study of animal relationships. Biol. Bull., **50**:73.

BOYDEN, A. A., and BAIER, J. G., JR. 1929. A rapid quantitative precipitin technic. Jour. Immun., **17**:29.

HEIDELBERGER, MICHAEL, and KENDALL, FORREST E. 1935. A quantitative theory of the precipitin reaction, III: The reaction between crystalline egg albumin and its homologous antibody. Jour. Exper. Med., **62**:697.

HEIDELBERGER, M., KENDALL, F. E., and SOO HOO, C. M. 1933. Quantitative studies on the precipitin reaction. *Ibid.*, **58**:137.

HEKTOEN, L., and COLE, A. G. 1927. The preparation and precipitin reactions of egg albumin and blood proteins of the domestic fowl. Jour. Inf. Dis., **40**:647.

HOOKER, S. B., and BOYD, W. 1934. The existence of antigenic determinants of diverse specificity in a single protein, II: In 2 natural proteins: crystalline duck egg albumin and crystalline hen egg albumin. Jour. Immun., **26**:469.

HOPKINS, F. G., and PINKUS, S. N. 1898. Observations on the crystallization of animal proteids. Jour. Physiol., **23**:130.

LAKE, G. C., OSBORNE, T. B., and WELLS, H. G. 1914. The immunological relationship of Hordein of barley and Gleadin of wheat as shown by the complement fixation, passive anaphylaxis, and precipitin reactions. Jour. Inf. Dis., **14**:364.

MARSHALL, J., and WELKER, W. H. 1913. Precipitation of colloids by means of aluminum hydrate. Jour. Amer. Chem. Soc., **35**:820.

SATOH, T. 1933. Ueber Präzipitintiter und Präzipitingehalt. Zeitsch. f. Immunitätsforsch. u. exp. Therapie, **79**:117.

TAYLOR, G. L., ADAIR, G. S., and ADAIR, MURIEL E. 1934. The precipitation reaction, optimal proportions, neutrality and maximal precipitation in mixtures of albumin and antiserum. Jour. of Hyg., **34**:118.

WOLFE, HAROLD R. 1935. The effect of injection methods on the species specificity of serum precipitins. Jour. Immun., **29**:1.

———. 1936. The specificity of precipitins for serum. *Ibid.*, **31**:103.

THE INFLUENCE OF THE FREQUENCY OF STIMULATION ON THE SLOW AND THE FAST CONTRACTION IN CRUSTACEAN MUSCLE

(Two figures)

C. A. G. WIERSMA AND A. VAN HARREVELD
William G. Kerckhoff Laboratories of the Biological Sciences,
California Institute of Technology, Pasadena, California

AS WAS shown by the authors (1936, 1938), most muscles of the extremities of decapod crustaceans have a double motor innervation, each muscle being innervated by two axons which have different functions. The thicker of the two fibers causes a fast, the thinner one a slow, contraction. In most cases both kinds of contraction need more than one nerve impulse before a mechanical response is obtained. The only exception found so far is the adductor muscle of the crayfish claw (van Harreveld and Wiersma, 1936, and Wiersma, 1937), in which a single impulse in the thick fiber elicits a strong twitch. Stimulating the thin fiber for the adductor muscle, a mechanical response can be obtained only after a number of impulses have reached the muscle. In *Blepharipoda occidentalis* a mechanical response was seen after stimulating the thick fiber with two induction shocks following each other with an interval of 2–10 σ (Wiersma and van Harreveld, 1938). Stimulation with two shocks at longer intervals does not elicit a mechanical response, but with repetitive stimuli a contraction is still obtained with intervals up to 50 σ. A single nerve impulse does elicit an action current of considerable size, however. It was found that in *Blepharipoda* the slow contraction was larger than the fast one up to frequencies of 50 per second. In the adductor of the walking leg of *Cancer anthonyi*, in which the fast mechanical contraction also needs at least two nerve impluses, the fast contraction is higher than the slow one only with frequencies above 100 per second. As these phenomena were considered of importance for the relation between the slow and the fast contraction, a number of experiments were carried out on this line to obtain further evidence.

EXPERIMENTS WITH *Blepharipoda*

The method used was the same as described in previous papers. The thick and the thin fiber were isolated and each placed on a pair of micromanipulated electrodes. By turning a double switch one or the other could be connected with a vacuum-tube stimulator, supplying short ($\frac{1}{2}$ σ) rectangular currents at various frequencies. The recording of the mechanical contraction was carried out by a light auxotonic lever to which a small mirror was fastened, reflecting a beam of light to the slit of the camera used for the recording of the electrogram. Since the distance between the mirror and the camera was 3 m., the recording was very sensitive and indicated even the slightest contraction. The muscle-action currents were led off with chloride-plated silver electrodes and recorded with a Matthews oscillograph.

Starting from a frequency of 50 per second, at which, as we have shown (1938), the mechanical contraction caused by either of the fibers is roughly the same in this

animal, lowering the frequency gives an increased difference between the fast and the slow contraction, the fast becoming weaker and weaker in relation to the corresponding slow contraction. It was found, however, that as long as there is a contraction on stimulation of the thick fiber this occurs rather quickly, whereas slow contractions become apparent only after prolonged stimulation when the frequency is quite low.

At a certain frequency the fast contraction is no longer demonstrable whereas the same stimulation still gives a very distinct slow contraction. Since the action potential caused by a single impulse is quite large, as stated above, the action currents of the fast contraction are of course very distinct. The action currents obtained from the slow contraction at these frequencies are so poorly facilitated that they are very small and difficult to record. Thus the paradoxical phenomenon was found that a muscle giving relatively high action currents on stimulation of the thick fiber does not show any contraction, whereas on stimulation of the slow fiber much smaller action currents were

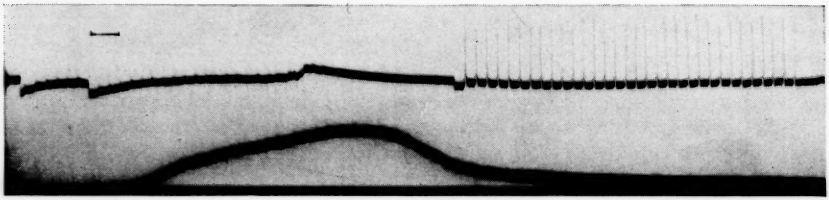


FIG. 1.—*Blepharipoda occidentalis*, adductor muscle, electro- (*a*) and mechanogram (*b*). Paradox with a stimulating frequency of 12 per sec. *A*, thin axon stimulated, action currents are very small, hardly detectable, contraction after a long latent period. *B*, stimulation of thick fiber, large action currents, no contraction, instead of which the relaxation of the slow contraction still continues. Notice the facilitation of the action currents. Time mark is $\frac{1}{8}$ of a sec.

elicited, causing, however, a very distinct contraction. We will refer to this phenomenon, which is illustrated in Figure 1 as the "paradox." It will be clear that this paradox can be obtained with any frequency between the minimum for the slow contraction and that of the fast contraction. In this animal the minimum for the slow contraction is about 10 per second, that of the fast 20 per second.

It was mentioned previously (1938) that the action currents of the fast contraction show facilitation when stimulated with frequencies of more than 1 per second. This facilitation depends only on the frequency of the stimulus; the higher the frequency the larger the facilitation. At the minimum frequency causing a mechanical response no sudden change of the facilitation is observed.

Another point which we investigated in this animal in relation to the mechanism of slow and fast contraction was latent facilitation. We have repeatedly pointed out that the slow action currents obtained after a short period of rest after a previous contraction grow faster than without a previous stimulation, as does the contraction. We confirmed this for the slow contraction in this animal and found it also to be true for the fast action currents. Working with a frequency at which no contraction took place (13 per second), we found that when two rather short stimulations were given with an interval of 1.5 seconds the first action current had in both cases the same height but the growth of the action currents was quicker during the second stimulation (Table 1). With a time

interval of 10 seconds this latent facilitation on a stimulation time of the same length had disappeared.

EXPERIMENTS WITH *Cambarus* AND *Astacus*

Under normal circumstances it is of course impossible to obtain a paradox in these animals, as the fast contraction needs only one nerve impulse. As was shown by Wiersma (1933), the mechanical contraction following a single nerve impulse can disappear when the stimulus is repeated a number of times with long intervals between successive stimuli, in which case the action current remains. The muscle then still contracts after

TABLE 1

Blepharipoda occidentalis: STIMULATION OF THE THICK AXON OF THE ADDUCTOR WITH A FREQUENCY OF 13/SEC.

	Height of Spike (in Mm.)					
	First Impulse	Second Impulse	Third Impulse	Fourth Impulse	Fifth Impulse	Ninth Impulse
First stimulation..... Rest 1½ sec.	12.2	13.1	14.0	14.5	14.9	16.0
Second stimulation..... Rest 5 min.	12.2	13.8	15.0	15.2	15.4	16.9
Third stimulation..... Rest 10 sec.	12.2	13.3	14.2	14.5	14.9	15.9
Fourth stimulation.....	12.3	13.0	14.0	14.5	15.0	16.0

two stimuli in close succession or during faradic stimulation. Wiersma found that the contraction disappeared in *Astacus fluviatilis* without any apparent change in the action current. We repeated these experiments both in *Cambarus* and in *Astacus trowbridgii*. In *Cambarus* the action currents diminish always, and sometimes even markedly, during the procedure, but in *A. trowbridgii* we were able to confirm his results as here there is often only a negligible reduction in their height. As was the case in *Blepharipoda*, there is no relation between the absence of the contraction and the reduction of the action current; if, for instance, the stimulation is continued after any trace of the contraction has disappeared, the action current still goes on diminishing at about the same rate as before (see Table 2).

In a preparation in which the mechanical contraction had thus disappeared, the paradox could be obtained. Stimulating such a preparation with frequencies between the minimum frequency at which the slow contraction appears (we found this to be about 5 per second for *Cambarus* and about 2 per second for *A. trowbridgii*) and that at which the fast contraction appears (under these circumstances about 15 per second), the action currents on stimulation of the thick fiber are very large but no contraction results, those on stimulation of the thin fiber small but a distinct contraction occurs, increasing, of course, with the frequency (Fig. 2). In this case there is even in *Cambarus* often no facilitation at all of the fast action currents, whether a contraction is elicited

or not, but sometimes some facilitation appears in those cases in which the action current had been considerably reduced during the stimulation series given to extinguish the fast twitch. In preparations in which the paradox can be obtained, contractions elicited

TABLE 2*

Astacus trowbridgii: STIMULATION OF THE THICK FIBER OF THE ADDUCTOR

Number of Impulse	Height of the Mechanical Contraction (in Mm.)	Height of the Action Current (in Mm.)
2	>50	22.5
3	>50	22.5
60	>50	21.5
90	15	21.0
120	2	21.0
150	1	21.0
180	.....	21.0
240	.....	20.0

* One induction shock every 5 seconds.

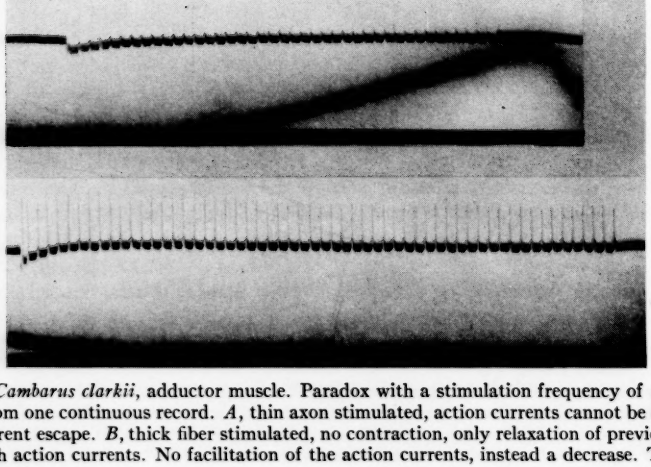


FIG. 2.—*Cambarus clarkii*, adductor muscle. Paradox with a stimulation frequency of 13 per sec. *A* and *B* are from one continuous record. *A*, thin axon stimulated, action currents cannot be distinguished from the current escape. *B*, thick fiber stimulated, no contraction, only relaxation of previous slow contraction, high action currents. No facilitation of the action currents, instead a decrease. Time mark is $\frac{1}{5}$ of a sec.

by a low-frequency stimulation of the thick fiber are smaller than the corresponding contraction on stimulation of the thin axon.

Another phenomenon which shows the relative independence of the mechanical contraction and the action currents is the following: When the fiber of the slow con-

traction is stimulated for a long time (frequency, e.g., 50 per second) an increase in the height of the action currents takes place in the beginning but is followed after a rather long period of constancy by a decrease. When the accompanying contraction is registered (strong isometric conditions) it is found that the contraction still increases long after the action currents have begun to diminish (see Table 3). Thus we find here that the mechanical contraction and the action current are influenced in opposite directions.

The fast action currents almost always diminish from the beginning; this reduction is especially quick with high frequencies, but here the mechanogram is much more affected by the fatigue and disappears long before the action currents are so small that they are no longer distinguishable.

TABLE 3*

Cambarus clarkii: FARADIC STIMULATION OF THE THIN FIBER OF THE ADDUCTOR

Time after Beginning of Stimulation (in Secs.)	Height of the Action Current (in Mm.)	Height of the Mechanical Response (in Mm.)
1	5.8	2.5
3	7.0	3.7
6	8.0	6.0
15	8.0	9.5
30	7.8	14.5
45	7.1	19.5
60	5.8	21.5
75	5.0	21.7
90	4.6	21.0

* Frequency 50 per second.

Investigating the slow and the fast contractions with high frequencies of stimulation, we observed that neither of them gave a normal response for any length of time (2 seconds) with frequencies above 200 per second. Nevertheless both showed contractions for short times on stimuli up to about 700 per second. Whereas the fast contraction increased in speed with the frequency, the slow contraction had a maximum speed at 200–400 per second.

It was of interest to note that in several preparations the shape of the fast contraction with a frequency of 50 per second was about the same as that of the slow contraction stimulated with 200 per second, both developing the same maximal tension (duration of stimulation 2 seconds).

EXPERIMENTS WITH *Cancer*

Whereas in *Blepharipoda* we were always able to obtain the paradox with fresh preparations and we have never failed to obtain it in *Cambarus* using the method described, it is rather astonishing that we have not been able to find the paradox in *Cancer anthonyi*. From the fact that in this animal the interval in which the fast contraction is lower than the slow one is relatively prolonged (Wiersma and van Harreveld, 1938), one would expect that on lowering the frequency the fast contraction would disappear first. On the contrary, we found that in fresh preparations stimulation frequencies so low that they do not elicit a slow contraction still cause a fast one. In relation to this it is

of importance that the range of summation intervals of two induction shocks, which when applied to the thick fiber cause a mechanical response, is much larger in *Cancer* (2 to more than 36 σ) than in *Blepharipoda* (2–10 σ). In correspondence with this the stimulation of the thick fiber causes contractions on lower frequencies of stimulation than in *Blepharipoda* (10 per second still gave a distinct contraction). This may explain the absence of the paradox in *Cancer*. We noticed particularly that the tension developed by the fast contraction at these very low frequencies increased only during the first few stimuli and then remained constant. With somewhat higher frequencies the main development of the tension of contraction still occurs initially, and only a small increase takes place with continued stimulation. On the other hand, the slow contraction continues to grow for a longer time and attains higher tensions at stimulation frequencies below 100 per second.

DISCUSSION

It is now almost certain that both the fast and the slow contractions can be elicited in every muscle fiber of a muscle with a double motor innervation. The anatomical evidence points strongly in that direction, and recently we have obtained further evidence for this, observing muscle fibers of the adductor muscle of *Cambarus* contracting as well on stimulation of the axon for the fast contraction as on that of the slow contraction. We shall report on this later.

The facilitation of the action current has been referred by Katz (1936) to an increase in the number of muscle fibers reached by the nerve impulse in the course of a faradic stimulation. According to this explanation the action currents of the individual muscle fibers can follow the all-or-none relation and the difference between the slow and the fast contraction in *Cambarus* (e.g.) would be that, on stimulating the fiber for the fast contraction all the muscle fibers are excited and respond with an action current, whereas on stimulation of the fiber for the slow contraction only a few muscle fibers would be reached by the first nerve impulse, some more by the second, etc. We consider the "paradox" as a phenomenon excluding this explanation of the increase of the action current. Especially conclusive are the experiments on *Cambarus clarkii* and *A. trowbridgii*. In these animals it was observed that the action currents accompanying the fast contraction, whether they cause a mechanical response or not, are all of the same (maximal) height. This would, according to this explanation, show that approximately all the muscle fibers are excited, but in the "paradox" do not show a contraction. If now the slow contraction was due to the same mechanism, it is inexplicable why the small number of fibers excited during a stimulation with low frequency—as is indicated by the low action currents—should cause a mechanical response. Our conclusion must be that the action currents evoked by the stimulation of the two motor fibers are an index or accompaniment of activities which are located in different places in the same muscle fiber.

This conception excludes the possibility that the action currents in the individual muscle fibers follow the all-or-none relation in its strict sense, as in each muscle fiber can be produced action currents of at least two different heights. We do not believe that these two action currents are constant in amplitude or other character. We consider the action current of the crustacean muscle as the index, not of a conducted, but of a local, process (compare the conclusions of Rosenblueth, Davis, and Rempel, 1936, concerning the first electrical response of the nictitating membrane of the cat).

Both the action currents of the slow and those of the fast contractions show a distinct facilitation in marine crustaceans. This resemblance is further increased by the fact that both mechanisms show latent facilitation. It is thus likely that, although the two processes differ in their localization in the muscle fiber and in the readiness of their facilitation, they are fundamentally similar in character. In *Cambarus* the facilitation of the action currents of the fast contraction is usually absent. The same can be stated for the action currents in preparations in which the mechanical response to a single nerve impulse has disappeared. Since the action currents in the latter case do not show facilitation, it is evident that the appearance of a mechanical response when the stimulus is repeated with a certain frequency cannot be ascribed to a change in the action potentials. It must be assumed, therefore, that a second kind of facilitation is present, which must be referred to processes interposed between the action current and the contraction.

It is likely that the same kind of facilitation exists in the slow contraction. This cannot be demonstrated in the beginning of a stimulation, since here a marked increase of the action currents by the facilitation is seen, and this effect might be chiefly responsible for the increase in the mechanical response. It was mentioned, however, that after some time the action currents start to decrease again (fatigue), whereas at the same time the mechanical contraction still continues to increase. Evidently this increase in the mechanical contraction is not caused by an increase of the action potentials but is to be referred to some other process of facilitation, located between the action current and the contraction.

We conclude that although the different steps in the transmission from the nerve impulse to the muscle fiber resemble each other closely in the slow and in the fast contraction, these processes must take place in different situations on the muscle fiber and must also show differences in their relative magnitudes.

SUMMARY

It is shown that on stimulation of the thick and the thin nerve fiber, innervating the adductor muscle of the claw of *Blepharipoda*, a range of frequencies can be found in which the thick axon gives large muscle action currents and no contraction, whereas the thin axon gives very small action currents but a good contraction. This paradoxical phenomenon can also be obtained in the adductor of the claw of *Cambarus*, when the twitch caused by a single nerve impulse in the thick fiber has disappeared. It was not obtained in the adductor of the dactylopodite of the walking leg of *Cancer*.

From this and other experiments the tentative conclusion is drawn that the fast and the slow contractions take place in the same muscle fibers, each having its own mechanism of transmission between nerve impulse and contraction. These may be in both cases qualitatively the same but quantitatively different.

LITERATURE CITED

KATZ, B. 1936. Jour. Physiol., **87**:199.
ROSENBLUETH, A.; DAVIS, M.; and REMPREL, B. 1936. Amer. Physiol., **116**:387.
VAN HARREVELD, A., and WIERSMA, C. A. G. 1936. Jour. Physiol., **88**:78.
WIERSMA, C. A. G. 1933. Z. vergl. Physiol., **19**:349.
———. 1937. Z. vergl. Physiol., **24**:381.
WIERSMA, C. A. G., and VAN HARREVELD, A. 1938. Jour. Exper. Biol., **15**. (In press.)

THE RESPONSE TO MECHANICAL SHOCK BY THE CERCARIAE OF BUCEPHALUS ELEGANS[1]

(One figure)

HARRY T. FOLGER AND LLOYD E. ALEXANDER
Fisk University

MECHANICAL shock has long been known to have a pronounced stimulating effect on many organisms, eliciting responses in some instances that are as clear cut and precise as those brought about by such agents as light or electricity. But, owing largely no doubt to the difficulty of controlling the magnitude of the stimulus with any degree of certainty, there is a dearth of exact knowledge concerning the effects of mechanical shock. Numerous observations have been recorded to be sure, but most of them have been merely incidental and have noted little more than that the organisms under consideration do respond to such a stimulus.

However, a few observations of a more detailed nature have been made. Several workers have noticed the suspension of movement that results when streaming cells of various plants are subjected to mechanical shock. Ewart (1903) found the protoplasmic streaming not only stops when cells of *Chara* and *Nitella* are so stimulated but that the time from the application of the shock until stoppage occurs—the reaction time—is longer after a light shock than after a heavy one and that movement remains in abeyance longer under the latter condition. Folger (1926) has reported that mechanical shock causes a cessation of protoplasmic flow in *Amoeba proteus* and that the same thing obtains with regard to the reaction time and period of quiescence as in streaming cells of *Chara*. Moreover, he found that a distinct refractory period follows the reaction. A certain amount of time must be allowed for recovery from the effects of a previous shock before the amoeba will respond to another, and a recovery which is not quite complete results in a reaction time that is longer and a period of quiescence that is shorter than after complete recovery.

Though there has been little or no disagreement in the observations on the effect of mechanical shock, the meagerness of evidence makes the study of other forms desirable. Most organisms are not suitable for work of this sort, as either they do not react at all or, if they do, the extent of the reaction is not easy to measure. The discovery of an animal, the cercaria of a flatworm parasite, which not only responds readily and consistently but with a reaction that can be accurately timed with a stop watch, has led to the investigation which is to be presented.

MATERIALS AND METHODS

The animals that were used in the following experiments were the cercariae of the gasterostome parasite, *Bucephalus elegans*, larval stages of which are passed in the gonad of the fresh-water clam, *Eurynia iris*. The cercariae emerge through the excurrent siphon of the clam and execute active swimming movements.

[1] Contribution from the Biology Laboratory of Fisk University. The authors are indebted to Dr. Arthur E. Woodhead for help and advice in the securing of experimental animals.

A large number of clams was collected in the early fall from the Huron River near Ann Arbor, Michigan. Infected individuals were isolated and kept in a cool room, in a large concrete tank, or imbedded in sand in glass aquaria. When animals were desired for experimentation, a few of these clams were transferred to finger bowls and put in a warm room. Within 24 hours there was usually an abundance of cercariae swimming about in the bowl.

A drawing of a cercaria is shown in Figure 1. As may be seen, it is of the fork-tailed type, the tail being cleft for almost its entire length. The two rami resulting from the bifurcation constitute the bulk of the animal. Locomotion is brought about by movements of the rami, which become extended so as to form a straight line and then curl

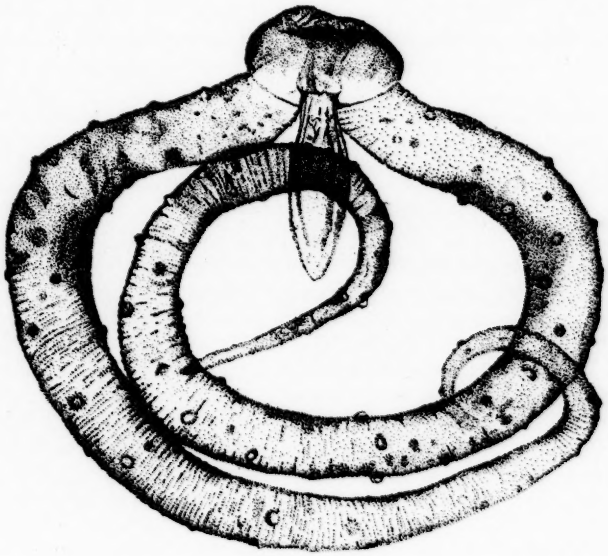


FIG. 1

up and at the same time shorten and thicken, assuming the position shown in Figure 1. When fully extended, the cercariae are from $\frac{1}{2}$–1 cm. in length. The rate of contraction and the extension of the rami depends on several factors, among them temperature and age, and there is considerable individual variation. At about 20° C. the rate is usually between 55 and 70 contractions per minute. A stimulus such as sudden illumination or mechanical shock causes a temporary cessation of movement.

The precise nature of the reaction to mechanical shock depends upon the intensity of the stimulus. If the shock is slight, it may have no effect, or it may cause the cercaria to cease progression through the water and the rami to undergo quivering motions, but without complete suspension of movement. A somewhat heavier shock causes the animal to become quiet, and in doing so the rami are generally folded. Very heavy shocks not only bring about cessation of movement but may cause the rami to remain in any

position, extended or contracted. In every instance in which movement stops, there is an appreciable reaction time, which, as used in this paper, consists of the time from the reception of the stimulus until the cessation of motion, without regard to the position of the rami.

The apparatus which was used in the experiments consisted of a narrow glass tube, 12.25 inches in length, supported in an upright position on a ring stand, and weights of the various sizes indicated in the text, made by cutting off appropriate lengths from wire nails. These weights, when dropped through the glass tube upon the deep depression slide on which the cercariae were put, brought about the shock. The slide was placed flat on the base of the ring stand in such a position that the falling weights would strike it midway between depression and end. The glass tube was clamped loosely near the top, with the lower part hanging freely, and the lower end almost touching the slide. On impact, the weight rebounded up into the tube, and a quick push against the latter threw it to one side, thus preventing it from again striking the slide and adding to the shock already produced. Care was taken in every test to make certain that the cercaria was swimming freely in the water and was not in contact with the glass. Time was measured with a stop watch. The reactions were sufficiently sharp to permit timing to one-fifth of a second.

EXPERIMENTAL RESULTS

Since *Bucephalus* cercaria react to mechanical shock after an appreciable reaction time, an obvious approach to a better understanding of the effect of this stimulating agent is an investigation of the relation existing between the intensity of the mechanical shock and the duration of the reaction time, for, as we shall see, the latter varies with the magnitude of the stimulus. The results of such an investigation are shown in Table 1. In this table are recorded the reaction times of 13 individuals, stimulated by shocks brought about by the fall of weights ranging in size from 177 mg. to 2,525 mg. Since, as already stated, each dropped a distance of 12.25 inches, the shock was proprotional to the size of the weight. So far as practical use is concerned, the foregoing weights represent extremes. It is shown in the table that only 2 animals out of 13 responded to the 177-mg. weight; a lighter one would have been even less effective. And a weight of only a little more than 2,525 mg. not only caused such a jar as to endanger the glass slide but often broke off the rami of the cercariae.

The behavior of individual No. 1 may be presented as a typical example illustrating the relation between intensity of shock and reaction time. In this animal the 2,525-mg. weight brought about a reaction in 0.8 seconds, the 2,025-mg. weight in 1.2 seconds, the 1,750-mg. weight in 1.2 seconds, the 1,040-mg. weight in 1.4 seconds, the 631-mg. weight in 2.4 seconds, the 501-mg. weight in 2.0 seconds. There were no responses to weights of 288 mg. and 177 mg.

Although not denoted in every instance, it is clear from this example that the reaction time tends to increase with a decrease in the force of the mechanical shock. Exactly the same thing is indicated in the reactions of the remaining 12 individuals, and, as shown in the lower row of Table 1, it is indicated especially clearly in the average reaction times of all of the animals. These averages, brought about by the fall of weights from the heaviest to the lightest, respectively, were 1.19, 1.44, 1.65, 1.70, 2.17, 4.38, 5.90, and 5.50 seconds. The decrease in the final instance is probably of no significance, as all except 2 of the animals failed to respond to so slight a stimulus.

While the behavior just described is certainly generally true, it is only fair to note certain exceptions. The reactions of individual No. 4 were obviously exceptional. This animal reacted to a weight 2,525 mg. in 1.8 seconds, to a weight of 2,045 mg. in 2.2 seconds, and to a weight of 1,750 mg. in 3.0 seconds. So far the reactions are as one would expect; that is, the reaction time increased with decrease in intensity of stimulation. However, stimulation by a still lesser weight, one of 1,040 mg., not only was not followed by a still further increase in reaction time but actually by a very decided decrease, the reaction time dropping to 1.0 second. Further reduction in the size of weights

TABLE 1*

SHOWING THE REACTION TIME FOLLOWING SHOCKS OF DIFFERENT DEGREES OF MAGNITUDE

Animal No.	Weight in Milligrams							
	2,525	2,045	1,750	1,040	631	501	288	177
1	0.8	1.2	1.2	1.4	2.4	2.0	No	No
2	1.0	1.4	1.0	2.0	1.4	2.0	5.0	No
3	1.2	2.0	1.4	1.4	2.0	1.4	8.0	5.0
4	1.8	2.2	3.0	1.0	1.2	1.4	No	No
5	1.0	1.0	1.0	1.2	2.0	1.8	No	No
6	1.0	1.4	1.4	2.0	1.2	15.0	No	No
7	1.0	1.2	1.2	1.2	3.0	No	No	No
8	1.4	1.0	1.2	1.4	2.0	2.0	No	No
9	1.0	1.0	1.4	1.4	3.4	3.6	No	No
10	1.0	2.0	2.0	2.0	No	No	No	No
11	1.2	1.4	1.6	2.0	3.0	4.4	4.4	6.0
12	2.0	2.2	3.0	3.0	2.0	12.0	8.0	No
13	1.0	1.0	2.0	2.2	2.4	2.6	4.0	No
Average	1.19	1.44	1.65	1.70	2.17	4.38	5.90	5.50

* The reaction time consists of the time from the application of the stimulus until the cercaria ceased to move. *No* indicates failure to respond. At least 3½ minutes intervened between tests.

brought about the customary increase in reaction time. The weight of 631 mg. caused a reaction in 1.2 seconds, and that of 501 mg. in 1.4 seconds. Folger (1925) noted similar sudden changes in the responses of *Amoeba* to light. Such irregularities in reaction are due to changes in the physiological state of the organism and by no means invalidate a conclusion, such as the one proposed in the first sentence of the preceding paragraph, which presupposes that the physiological state will remain constant.

Doubtless many factors besides intensity of shock affect the reaction time, but only one other has been investigated—the effect which a given shock has on the reaction to another shock which follows quickly after it. The results of this study are set forth in Table 2. Nineteen different animals were used in the experiment, the procedure of which consisted simply in applying a series of stimulations to the chosen animals, with certain intervals, ranging from 60 seconds to 15 seconds, between them. As indicated in the table, animals Nos. 1 and 3 were subjected to nine successive shocks, with intervening periods of 50 seconds. Both animals reacted in every instance, and in both the reaction time remained practically constant, at about 1.2 seconds. Number 2 gave exactly the

same results up to the sixth reaction, when it was accidentally killed. Animals 4, 5, 6, and 7 were treated as the preceding animals, except that intervals of 30 seconds were allowed between shocks. Numbers 4, 5, and 7 continued to respond for nine or ten times without an appreciable change in reaction time. They would probably have responded for a longer time had they received further stimulations. Number 6 failed to respond to the third shock. Animals 8, 9, and 10 were allowed 25 seconds between shocks. Number 8 failed to respond after the third stimulation, No. 9 after the fourth stimulation, and No. 10 after the sixth stimulation. And not only was there finally a cessation of response but the reaction time decreased very considerably before the responses

TABLE 2*

SHOWING EFFECT OF INCOMPLETE RECOVERY ON THE REACTION TIME

Reaction No.	Time Allowed for Recovery																									
	60 Sec.				30 Sec.					25 Sec.				20 Sec.				15 Sec.							
	Animal No.			Ave.	Animal No.				Ave.	Animal No.			Ave.	Animal No.			Ave.	Animal No.						Ave.
	1	2	3		4	5	6	7		8	9	10		11	12	13		14	15	16	17	18	19	
1	1.0	1.2	1.4	1.2	1.4	1.0	1.0	1.0	1.0	1.2	2.0	1.6	1.6	1.6	1.2	1.0	1.3	1.6	1.4	2.0	1.4	1.4	1.0	1.8
2	1.2	1.2	1.0	1.1	1.4	1.2	1.0	1.0	1.2	1.2	1.0	1.0	1.1	1.0	0.8	1.0	0.9	1.0	1.4	1.4	1.0	0.8	0.8	1.3
3	1.0	1.2	1.4	1.2	1.4	1.0	No	1.0	1.1	0.8	0.8	1.0	0.9	0.8	0.6	0.8	0.7	1.0	1.2	1.2	0.8	1.0	No	1.0
4	1.0	1.2	1.2	1.1	1.4	1.0		1.0	1.1	No	0.8	0.8		0.6	0.6	0.8	0.7	No	No	No	No	No		
5	2.0	1.0	1.4	1.5	1.4	1.0		1.0	1.1		No	0.8		No	No									
6	1.0	1.2	1.4	1.2	2.0	1.0		1.0	1.3			0.6												
7	2.0		1.2	1.6	1.4	1.0		1.0	1.3			No												
8	1.0		1.2	1.1	1.4	1.0		1.2	1.2															
9	1.0		1.2	1.1	1.2	2.0		1.2	1.5															
10					1.4																			

* Reaction times given in vertical columns and expressed in seconds. Note that 30 seconds was sufficient for complete recovery, as indicated by a reaction time that remained constant after repeated tests, and that 25 seconds or less did not permit complete recovery, as indicated either by a reaction time that was shorter than usual or by failure to respond.

stopped. Thus, at the first shock the reaction time of cercaria No. 8 was 1.2 seconds, but at the third shock it was only 0.8 seconds. The reaction time of No. 9 fell from 2.0 seconds at the first stimulation to 0.8 seconds at the third. Cercaria No. 10 reacted to six successive shocks, with reaction times expressed in seconds as follows: 1.6, 1.0, 1.0, 0.8, and 0.6. The next group, consisting of animals Nos. 11, 12, and 13, were allowed 20 seconds between stimulations. The first two ceased to respond after the fourth shock, the third after the fifth shock. The average reaction times of the 3 animals amounted the 1.3 seconds, 0.9 second, 0.7 second, and 0.7 second for four successive stimulations. The final group of 6 animals were allowed 15 seconds between shocks. None of them responded more than three times, and No. 19 only twice. The average reaction times were 1.8 seconds, 1.3 seconds, and 1.0 second.

From these results it is apparent that at least 30 seconds are necessary for complete recovery from the effects of a mechanical shock and that incomplete recovery is made evident either by failure to respond to another shock or by a reaction time that is

shorter than that after complete recovery. Furthermore, there is clearly a cumulative effect. A series of stimulations, with an insufficient recovery period between, results in a steady decrease of the reaction time until finally there is no reaction at all.

DISCUSSION

The increase in reaction time with decrease in the force of the mechanical shock that has just been observed in the reactions of cercariae is exactly what one would have expected. As mentioned in the introduction, this stimulating agent is known to have a similar effect on some other organisms, and the same thing is true in the reactions of various organisms, including the cercariae of *B. elegans* (Bevelander, 1933),[2] to other forms of stimuli, notably light. But the result of insufficient time for recovery is rather surprising. A number of investigators have observed that such a condition may have a very decided effect on the reaction time, but in nearly every instance, both in the case of mechanical shock and in the case of light, the effect has been manifested by an increase in reaction time. Here there is just as decidedly a decrease. Just why *Bucephalus* cercariae should behave differently from other organisms in this one respect is not certain, especially as their reactions are in accord in other respects. It is possible, however, that they do not behave so differently as appearances indicate.

A well-known fact of physiology is the temporary loss of sensitivity which follows stimulation of a nerve, giving rise to the interval known as the refractory period. This period is divisible into two parts—the absolute refractory period, which follows immediately after stimulation and during which there is a complete loss of excitability, and the relative refractory period, during which the nerve will respond to stimulation but not so readily as ordinarily. It is a significant fact that distinct refractory periods, both absolute and relative, are known to follow the reactions of a number of organisms to light and mechanical shock. *Amoeba*, for example, responds to both of these stimulating agents by a cessation of movement. It will not immediately respond to another stimulus at all; and, even after it does begin to respond, it is at first less sensitive than usual, as indicated by an increase in the reaction time. But another thing which has been observed in nerve is that at the beginning of the relative refractory period there is a supernormal phase, during which the nerve is not less but more excitable than usual. Folger (1925) has observed a supernormal phase in the response of *Amoeba* to light. While feeding, an amoeba refuses to respond to light at all. A minute or so after it has fed, it is considerably less sensitive than usual, but immediately after feeding it responds much more readily than at any other time. It appears not at all improbable that the increase in sensitivity in the cercariae of *B. elegans*, which is caused by repeated shocks with only a small time interval between and made evident by a decrease in reaction time, is similar to the supernormal phase in the response to light which follows feeding in *Amoeba*.

SUMMARY

1. The cercariae of *B. elegans* react to mechanical shock by a cessation of movement, which takes place after an appreciable reaction time.

2. The reaction time varies inversely with the magnitude of the shock. This agrees with the reactions of various other organisms to both mechanical shock and light.

[2] In response to light the reaction time increases with decrease in luminous intensity.

3. After cercaria has reacted to a mechanical shock, it will not immediately react to another. About 30 seconds are required for complete recovery from the effects of such a stimulation. Recovery may be partial, as indicated by a reaction time that is less than that obtained after complete recovery. This does not agree with the reactions of other organisms, where incomplete recovery from the effects of mechanical shock or light is indicated by an increase in reaction time.

LITERATURE CITED

BEVELANDER, G. 1933. Response to light in the cercariae of *Bucephalus elegans*. Physiol. Zoöl., **6**:289–305.

EWART, A. T. 1903. On the physics and chemistry of protoplasmic streaming in plants. London: Oxford Press. Pp. 131.

FOLGER, H. T. 1925. A quantitative study of reactions to light in amoeba. Jour. Exper. Zoöl., **41**:261–90.

———. 1926. The effects of mechanical shock on locomotion in Amoeba proteus. Jour. Morph. and Physiol., **42**:359–70.

THE NUTRITIONAL REQUIREMENTS OF BLATTELA GERMANICA

(Three figures)

C. M. McCAY
Animal Nutrition Laboratory, Cornell University, Ithaca

IN SPITE of the wealth of available material, insect nutrition has been given little attention in contrast to that of such vertebrates as the rat.

Trager (1935) has recently summarized the evidence showing that a number of insect species have specific requirements for vitamin-like compounds found in such materials as yeast and wheat germ. However these substances seem to differ from B_1 and lactoflavine although some insects seem to require some such fractions. Trager finds mosquito larvae need fractions found in liver extract and yeast products.

The webbing clothes moth also requires part or all of the B-complex for growth and development (Crowell and McCay, 1937).

The cockroach (*Blattela germanica*) has no vitamin requirements according to the findings of Wollman (1926). This author generalized upon his findings, claiming that insects are distinguished from the higher animals in their ability to grow and reproduce without such accessory factors. Zabinski (1929) also studied the requirements of this insect and concluded they required no other source of nitrogen than glycine.

These remarkable claims for an insect that is an unwelcome guest in so many nutrition laboratories made this ancient pest especially attractive for study.

BREEDING AND REARING A STOCK OF COCKROACHES

For a period of several years various cages were tried for nutrition experiments with cockroaches. The design shown in Figure 1 has proved very satisfactory both for testing a given diet upon isolated individuals and for studies with groups. In Figure 2 is shown a satisfactory breeding cage which is used to provide an ample supply of stock animals.

The test cage of Figure 1 is a half-pint milk bottle fitted with a three-hole cork stopper. Three tubes of 0.9 cm. glass tubing provide an air hole, access to a vial containing a piece of cotton soaked in water, and another vial holding the diet to be tested. The food can also be placed in the bottom but it seems more liable to attacks by molds. Numerous studies in the course of the last six years have indicated that this cage provides adequate ventilation and nearly optimum humidity. Among the types of cages tested have been those constructed of very fine wire screening and open on all sides. The humidity was controlled in such cages. These have little advantage and many disadvantages. Other studies have been made with several vents in the cork stopper of the milk bottle. All of these have indicated that the cage of Figure 1 is probably the most satisfactory devised thus far.

For producing large numbers of stock animals the museum jars of Figure 2 have proved useful. The water in such cages is provided by a funnel filled with a cotton plug and set in a beaker of water. This prevents the drowning of the insects and fouling of the water. The water is easily renewed by pouring from a wash bottle at intervals of several days. The food supply is placed in a vial or a small beaker.

For six years a stock diet of equal parts of dry skim milk and ground whole wheat has proved adequate for the entire life-cycle of the species. From time to time various other food substances have been tried but no additional advantage has been found.

For rapid growth and reproduction a temperature of twenty-five degrees or more is desirable. For testing foodstuffs an old refrigerator equipped with a thermostat has proved very satisfactory in providing a constant temperature.

The growth rate of this species is similar to that of the rat. It is easily followed by periodical weighings upon an analytical balance since the range of weight from the young to the largest adult varies between one and a hundred milligrams. This insect is very sensitive to ether anesthesia. This provides a convenient method of handling.

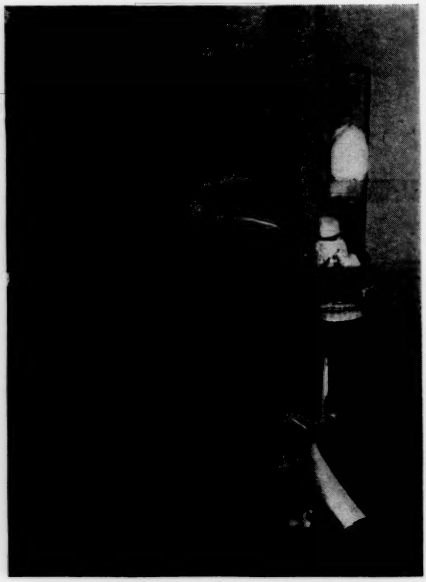


FIG. 1.—Cage for nutrition studies with cockroaches. Food can be placed either in the vial on the sidetube or in the bottom. The upright vial has a cotton plug saturated with water. The cloth-covered tube provides air.

For securing test animals the first step is the isolation of females bearing egg sacs. This is done by moistening the cloth cover of the stock cage with ether. The females are then placed in test bottles using a pair of light forceps. These are examined at daily intervals. The date of hatching of the young is recorded. They can then be placed under light ether anesthesia and distributed to test bottles. Ten individuals per bottle afford a very satisfactory group. Special studies have shown that this anesthesia does not modify the growth-rate even if repeated at weekly intervals.

The productivity of the females can be illustrated by a single experiment. Three females bearing egg sacs were placed in a stock cage. Five months later this cage contained seventy adults, many of them bearing mature capsules. This suggests the possibility of using this species for population studies with results that might be more applicable to higher animals than those made with *drosophila*, since the food habits are more nearly like those of vertebrates.

THE CONVERSION OF ELEMENTS FROM FOODSTUFFS INTO BODY TISSUES BY INSECTS

Various species differ widely in their ability to convert the chemical elements of foodstuffs into body tissue or secretions. Lusk (1928) notes that the cow can secrete 5,378 calories of energy in the form of milk, while its body burns 11,464 calories as measured by the calorimeter. Armsby and Moulton (1925) has discussed the numerous variables, such as the rate of growth, individual variability, and species differences that determine the efficiency of higher species in building the chemical elements of foodstuffs into body tissues. From the data of Palmer and Kennedy (1931) rats in the course of their growth

seem able to convert about 30 per cent of the nitrogen of their food into that of their body. The accumulation of errors in such a calculation may amount to as much as 30 per cent of the above value.

A few studies of the ability of lower vertebrates to convert foodstuffs into body tissues indicate an efficiency superior to the best of the higher vertebrates. Brook trout are somewhat superior to such species as swine or chickens (McCay, 1937). In some time periods of 4 weeks trout may even require less than a gram of dry foodstuff to produce

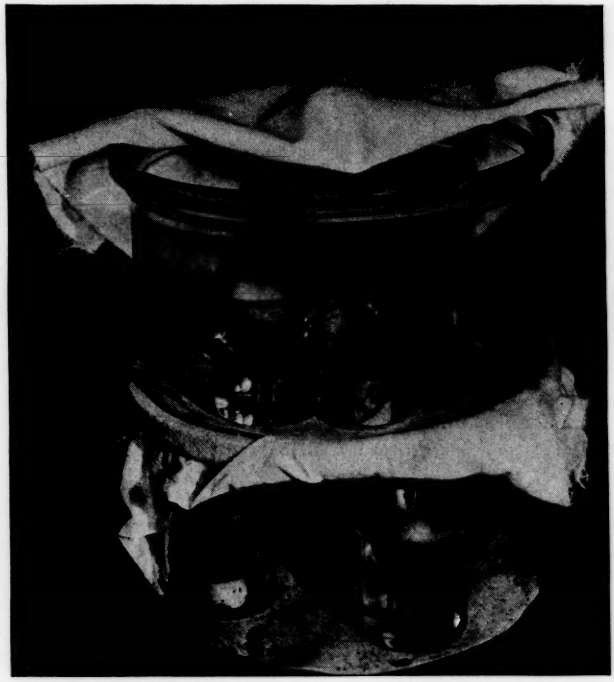


FIG. 2.—Stock-breeding cages. The one above is ready for use while the one below contains insects. The funnel in the beaker of water is stuffed with moist cotton. The feed is in the vial. The cloth covering moistened with ether provides the anesthetic when females are removed.

a gain of 1 gram in live weight. Poikilothermes may have some advantage in the conversion of foodstuffs, since they have no body temperature to maintain.

Since no data are available concerning the conversion of elements by insects, the present study was undertaken. Conditions were favorable for such a determination since a satisfactory dry diet had been developed that permitted an insect to pass through its entire life-cycle without limitations due to the quality of the diet. The use of a single diet may permit an accurate estimate of the utilization of calories, but the determination of the efficiency of the conversion of other elements in such a diet cannot be optimum

unless such elements are present in the diet at the lowest levels that just permit optimum growth. There is no reason to believe that this was the case in the present study. For this reason the values obtained are accurate for the diet under consideration but are not optimum ones except in the case of the calories concerned.

The efficiency of transformation of chemical constitutents into body tissues can be measured in *B. germanica* by weighing a known allowance of foodstuff, allowing groups to grow to maturity upon it until all the foodstuff is consumed, and finally analyzing the bodies of the insects produced upon the foodstuff. The greatest error in this procedure is due to the foodstuff retained in the intestinal tract. This structure is so delicate that it is impossible to wash it free from food residues in extensive numbers of individuals.

The following procedure was employed: Three groups of young insects, one to two days after hatching, were confined in separate jars after their body weights were recorded and a sufficient number had been taken for analysis. Into each jar was placed a vial of foodstuff that had been carefully weighed. When the insects in each jar had attained adult size they were removed, weighed, and preserved for analysis. Into each jar a new group of young was introduced and the procedure repeated until all the foodstuff was consumed.

From this study the weight and composition of both the foodstuff and the insects produced were determined by analysis. In the preliminary preparation the foodstuff was carefully moistened with 95 per cent alcohol and then dried carefully *in vacuo*. A sample was sealed for analysis. The foodstuff consisted of a mixture of equal parts of dried skim milk and whole ground wheat.

Each jar produced nearly three generations of mature insects. The following analytical procedures were employed: nitrogen—Barrett and Jones (1932); ash—ignition in the muffle furnace; calcium—Morris, Nelson, and Palmer (1931); phosphorous—Youngberg and Youngberg (1930); lipids—extraction of the dried material with ethyl ether in the Soxhlet apparatus for 16 hours; I. No.—Yasuda (1931).

In order to reduce the error for the food residues of the intestinal tract to a minimum, the insects were fasted for 24 hours before killing in each case.

In order to determine the error that could be expected from the food residues, groups of adult insects not involved in these studies were killed without fasting and also after intervals of 1–5 days without food. The longest period may cause death if individuals are isolated and cannibalism if they are associated. Fifty representative adults from each group were weighed, the posterior end of the body snipped, and the intestinal tract removed by pulling the head. This tract was then removed. The tracts and contents were dried and weighed separately from the bodies. With no period of fasting the tract and residues were equal to 9 per cent of the body weight while after 5 days of fasting this value fell to 4.6 per cent. In the latter case there was little food residue and this probably represents the intestinal tract. The error from neglecting food residues in insects that have fasted for one day must then be less than 5 per cent.

The analytical data from these studies are assembled in Tables 1 and 2. These data indicate clearly that the insect is very efficient in the conversion of foodstuffs compared to higher animals. This species is able to build nearly a unit weight of the living body from a unit of dry foodstuff. It is able to synthesize fat since the bodies of the insects contained about three times as much lipid as the foodstuff, ingested. Also, this body fat had only about half the iodine number of that ingested, which affords evidence for a characteristic body fat.

In these experiments about 60 per cent of the nitrogen, 10 per cent of the calcium and 25 per cent of the phosphorus of the diet were converted into body tissue. Analytical values are not available for the chitin of the insect and the carbohydrate of the diet, but estimates indicate that about 40 per cent of the calories in the diet were built into the body of the insect.

THE FEEDING OF MIXTURES OF PURIFIED CONSTITUENTS

In preliminary trials it was found that this species could not grow upon mixtures of casein, starch, cellulose, sugar, lard, and mineral mixture unless this mixture were supplemented with yeast.

TABLE 1

THE CONVERSION OF FOODSTUFF INTO BODY TISSUE BY INSECTS

Expt. No.	Wt. Insects Added (Grams)	Wt. Insects Removed (Grams)	Dry Wt. Insects Removed (Grams)	Per Cent H_2O	Calcd. Dry Wt. Increase (Grams)	Dry Food Consumed (Grams)
M-1	0.5002	8.8483	3.1359	64.6	2.9587	9.3418
M-2	0.6026	7.8599	2.8077	64.3	2.5922	8.2832
M-3	0.8766	8.8296	2.9789	66.3	2.6835	8.5769

TABLE 2

COMPOSITION OF THE INSECTS AND FOODSTUFF IN PERCENTAGE OF DRY WEIGHT

Expt. No.	N	Ash	P	Ca	Ether Sol.	I. No. of Fat
M-1	8.86	4.27	0.67	0.26	15.6	57
M-2	8.65	4.56	0.66	0.25	16.2	55
M-3	8.82	4.60	0.72	0.36	17.1	57
Foodstuff	4.48	4.63	0.80	0.72	0.86	111

This indicated there was a need for supplementary factors (McCay, 1933). In one study a group was maintained for 2 months upon the synthetic diet without supplements and then grew normally when transferred to the stock diet. This indicated that growth could be resumed after a period of retardation.

In order to check the first findings the diets shown in Table 3 were designed. The salt mixture (S.M.) was that of Osborne and Mendel (1919). The other constituents had been extracted for use in vitamin A testing with rats.

This experiment was started by placing twelve individuals in each bottle with the respective diets. The mean weight at the time they were placed in the bottles was 3.8 mg. The bottles were then placed in the incubator at 25° C. for 60 days. They were examined at intervals of 2 weeks and the growth noted, but they were not disturbed. At the end of 60 days they were etherized, weighed, and pasted to cards. The photograph, Figure 3, shows the relative sizes of the members of the different groups. In this case the differences are so obvious that it is hardly necessary to refer to the column of mean weights in Table 3.

In addition to confirming the first conclusions that cockroaches need some vitamin supplements, these data indicated some interesting lines of attack. In the first place, Group 20 was inferior to Group 14. These groups were not strictly comparable, however, since the diets differ in composition, but they suggest that gelatin may be less satisfactory than casein as a source of protein. This problem is subject to solution with insects as well as rats but is difficult, because the cockroach can probably select yeast cells from the remainder of the food while the rat cannot. This makes it necessary to work with soluble vitamin extracts instead of particles so that no opportunity is afforded for selection by the insect. It is quite possible that gelatin may be an incomplete protein in Diet 20 and that there may be essential amino acids for insects as well as rats. This

TABLE 3

THE COMPOSITION OF DIETS WITH THE FINAL NUMBER ALIVE AND THE MEAN WEIGHT

Diet No.	Composition	Number Alive after 60 Days	Final Mean Weight in Mg. after 60 Days
5	Whole wheat 50, dry skim milk 50	10	lost
10	Casein 5, S. M. 5, sucrose 90	4	10.3
12	Casein 40, S. M. 5, sucrose 55	7	12.7
13	Casein 40, S. M. 5, starch 55	7	13.4
14	Casein 40, S. M. 5, starch 45, yeast 5, C. L. O. 5	7	62.0
15	Casein 40, S. M. 2, starch 58	8	14.3
16	Zein 20, S. M. 5, sugar 75	8	13.7
17	. . . . , S. M. 5, sucrose 95	11	7.4
18	Gelatin 5, S. M. 5, starch 90	10	13.8
19	Gelatin 20, S. M. 5, starch 75	8	12.7
20	Gelatin 20, S. M. 5, sucrose 65, yeast 5, C. L. O. 5	9	45.6

experiment might easily fail to show this if the insect ate enough yeast cells to satisfy its amino acid requirement.

These data indicate that Diet 17 was the most effective in preventing growth. In 60 days this group only grew as much as they would ordinarily grow in 4 days on a complete diet. On the other hand, a large percentage of this group survived.

Unfortunately, the mean weight of the group fed the stock diet of wheat and skim milk was lost, but during the entire experiment this group grew more rapidly than any other. This mixture of natural foodstuffs is superior to the synthetic diet with its addition of cod liver oil and yeast. In all experiments upon this stock diet with its base of whole wheat it has been noted that the cockroach refuses to ingest much of the cellulose. This is sorted out and left behind as the diet is consumed.

These experiments also indicate that cannibalism will not interfere seriously with assay experiments in this case. Even Group 10, which ended with only a few insects, made little gain in weight.

THE ACCESSORY FACTORS RESPONSIBLE FOR GROWTH

In a second series of experiments the earlier results were checked with modifications. New diets were devised. The composition of these diets is shown in Table 4. The con-

stituents of these diets were purified in the same manner as those used previously. Unfortunately, a number of other experiments in this series were lost through an accident.

Diets 26, 27, and 28 were designed to determine if the fat-soluble vitamins alone were sufficient for growth. Since Diet 26 gave poor growth compared to 27 and 28, this furnishes additional evidence that some factor in yeast is essential. On the other hand, Diet 37 was satisfactory for growth although it contained little fat while 38, which had ample fat but no yeast, failed. This experiment is of special interest because the diet containing the lard tended to mold but this mold was not used in supplying a

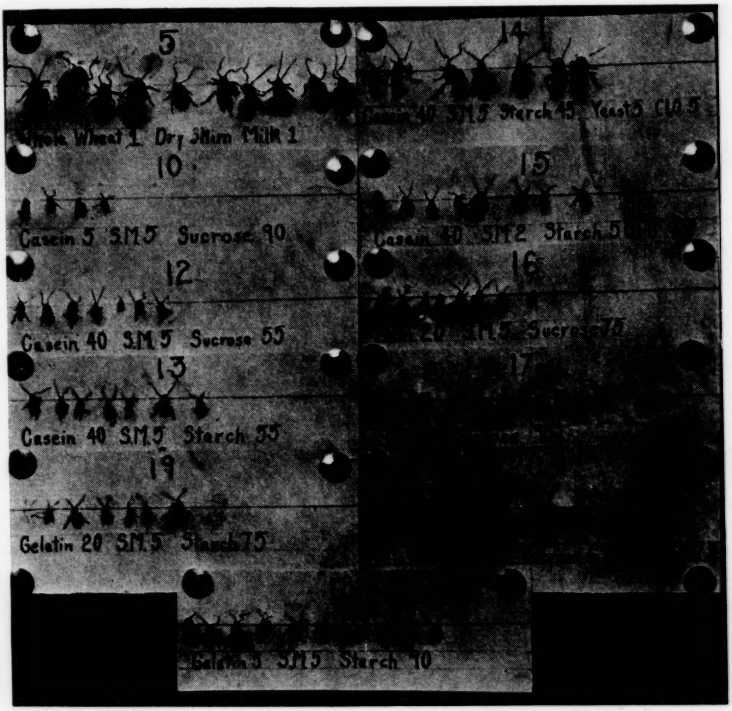


FIG. 3.—Cockroaches of the same age after being fed various diets for sixty days

growth factor. Number 37 indicates that vitamin studies with this species can be made with diets low in fat. This is especially desirable because of mold growths upon fat-rich diets.

Diets 39, 40, and 41 represent another attempt to determine if the quality of the protein is of importance to an insect. Owing to difficulties with molds this must be repeated, but this preliminary trial indicated that casein was the most satisfactory. In these experiments an alcoholic extract of yeast was used to avoid the danger of protein selection from yeast cells. Of the three proteins tested, casein was better than gelatin and this in turn proved better than zein. These experiments also provide a starting-point for de-

termining which B fraction is essential for the cockroach. The alcoholic extract permitted good growth, but this experiment was not carried through the entire cycle to include reproduction.

The combined evidence from all these experiments indicates that this species has little if any need for vitamins A or D but that there is a requirement for some of the factors of yeast. In no case was growth upon synthetic diets equal to that upon mixtures of dried skim milk and wheat.

An additional series of sixty experiments that will not be reported in detail indicated that extracts of yeast made with 95 per cent alcohol were more potent as supplements than either water extracts or those of water-alcohol mixtures.

TABLE 4

DIETS VARYING IN THEIR CONTENT OF YEAST AND IN THE QUALITY OF PROTEIN

Diet No.	Composition	Growth Rate
26	Casein 20, starch 62, S. M. 3, lard 10, yeast 0, C.L.O. 5	poor
27	Casein 20, starch 59, S. M. 3, lard 10, yeast 3, C.L.O. 5	good
28	Casein 20, starch 55, S.M. 3, lard 10, yeast 6, C.L.O. 5	good
37	Casein 20, starch 72, S. M. 3, lard 0, yeast 5, C.L.O. 0	good
38	Casein 20, starch 67, S. M. 3, lard 10, yeast 0, C.L.O. 0	poor
39	Zein 20, starch 62, S. M. 3, lard 10 (Alc. Ext.), C.L.O. 5	poor
40	Gelatin 20, starch 62, S. M. 3, lard 10 (Alc. Ext.), C.L.O. 5	moderate
41	Casein 20, starch 62, S. M. 3, lard 10 (Alc. Ext.), C.L.O. 5	good

ATTEMPT TO ASSAY ALCOHOLIC EXTRACTS OF YEAST

An attempt was now made to assay the alcoholic extract of yeast. Five hundred grams of dry yeast were extracted in a continuous extractor on the steam bath with 2,500 cc. of 95 per cent alcohol. This extract was stored in a dark bottle in the refrigerator.

For a basal test diet a mixture of casein 30, salt mixture 4, and starch 66 was used. (The salt mixture was that of Osborne and Mendel.) Five grams of this diet were prepared for feeding by sprinkling a given amount of the alcoholic extract over it and evaporating it dry *in vacuo* at 65° C. This was then ground in a mortar to a powder.

Three bottles of ten insects each were used for the first three diets and eight bottles of ten each for No. 104.

The mean weight of the insects in each bottle was determined at the beginning of the experiment and two months later. The results recorded in Table 5 show conclusively the effect of the highest level of yeast extract in the growth of No. 103. The lower levels gave results similar to the basal diet without supplements. One cubic centimeter of this alcoholic extract per gram of basal diet (No. 103) stimulated growth to about half adult size in the course of 2 months. This growth was not maximum compared to that obtained with the stock diet of wheat and milk, but it showed the stimulating effects of the extract quite clearly.

Two weeks after this experiment with casein was started, another was begun using gelatin instead of protein in the basal diet.

The same number of groups was employed for each diet except that ten groups of ten each were used for No. 108. The mean weights for these are recorded at the end of

2 and 3 months. Here again there is a marked stimulus to growth in the case of those allowed a supplement of 1 cc. of alcoholic extract per gram of basal diet (No. 107). There is little difference at the end of 2 and 3 months, indicating the shorter period was adequate for preliminary testing of extracts.

TABLE 5

THE EFFECT OF SUPPLEMENTING CASEIN AND GELATIN DIETS WITH DIFFERENT LEVELS OF ALCOHOLIC EXTRACT OF YEAST AND EXTRACT WITH MUCH OF THE NITROGEN REMOVED

Diet No.	Supp. in Cc. per 5 G. Feed	Mean Wt. Start (Mg.)	Mean Wt. 2 Mo. (Mg.)	Mean Wt. 3 Mo. (Mg.)
	"Casein" EtOH Exts. Yeast			
104	0.0	2.5	7.6	.....
101	0.1	3.5	8.3	.....
102	1.0	3.4	9.2	.....
103	5.0	3.3	24.4	.....
	'Gelatin" EtOH Exts. Yeast			
108	0.0	1.9	5.5	7.3
105	0.1	2.3	5.9	6.2
106	1.0	2.2	8.2	9.0
107	5.0	2.2	16.5	18.2
	"Casein" Pb $(AcO)_2Na_2CO_3$ Pptd. Exts. Yeast			
104*a*	0.0	4.9	10.2	14.2
110	1.0	4.6	10.1	18.6
111	5.0	4.6	11.9	20.4
112	20.0	5.0	18.0	35.1
	"Casein" EtOH Ext. Yeast			
103*a*	5.0	5.4	12.0	46.7

The analysis of the yeast extract used above gave the following values per cc.:

N = 0.039 mg.
P = 0.009 mg.
Dry matter = 9.6 mg.
Amino N = 0.1–0.2 mg. (higher value after 30 mins. in the Van Slyke App.)

To remove part of this nitrogen, 300 cc. of the alcoholic extract was treated with 3 cc. of saturated lead acetate followed by 3 cc. of 10 per cent sodium carbonate. This was filtered, centrifuged, and then treated with H_2S. After filtration it was made slightly acid with HCl and again saturated with H_2S. The filtrate from this procedure was re-

duced in volume on the steam bath, neutralized with NaOH, and finally made to a volume of 300 cc. with EtOH.

About one-fourth of the original nitrogen was still left in the solution while about three-fourths was removed by this precipitation.

To test the filtrate after treatment with mercuric acetate and sodium carbonate, three levels of the filtrate were fed as supplements to the casein basal diet used in 104. One, five, and twenty cubic centimeters of the filtrate were dried on 5-gram samples of basal diet (Nos. 110, 111, and 112). For comparison Diets 104*a* and 103*a* were used. The former contained no supplement and the latter 5 cc. of the original alcoholic extract. These diets with extract supplements are termed Nos. 110, 111, and 112.

These insects were weighed at monthly intervals for 3 months. The mean weights and the number alive are given in Table 5. These results indicate that the filtrate from the mercuric-acetate–treated solution was still potent in stimulating growth but much less effective than the original alcoholic extract of yeast. The development of mature adults upon the diet containing the yeast extract (103*a*) in contrast to that containing none (104*a*) was evident at the time of the last weighing.

The removal of much of the nitrogen from the alcoholic extract may prove useful in future work in testing the need for essential amino acids.

COMPARISON OF DIFFERENT LEVELS OF B_1 FED TO INDIVIDUALS AND TO GROUPS

In order to test a potent source of B_1 at various levels and at the same time to determine if groups instead of individuals can be used for such assays, the following experiments were run.

The same basal diet with casein as a protein source as that used previously was supplemented with B_1 concentrate from rice polishings (Burroughs, Welcome and Company). It was included in the diet at levels of 0, 0.05, 0.5, and 5 per cent. A positive control fed the stock diet was included in the series. Three bottles of ten individuals each were used for each diet as well as ten insects in individual cages. These were weighed at the beginning, the tenth day, and one month. Table 6 gives a summary of results.

These data indicate that there is little advantage in using individuals in preference to groups at the present state of testing. The conclusions would have been the same in either case.

No specific response to B_1 concentrate was obtained whatever the level. The addition of B_1 to the basal diet makes little improvement and the growth rate does not compare favorably between synthetic diets and natural stock diet. The basal diet is apparently highly deficient in factors other than B_1.

THE ETHER-SOLUBLE PORTION OF THE ALCOHOLIC EXTRACT

Four hundred cubic centimeters of the alcoholic extract were evaporated to a syrup. This was extracted with ethyl ether. A fine white powder and an orange paste remained behind. The white powder dissolved readily in EtOH but the syrup only dissolved after considerable boiling.

For this test insects from Diet 108 that had grown little were selected. It was assumed that these would resume growth if the extracts were potent and fail if they were not.

Neither the alcohol nor the ether-soluble fractions stimulated growth in the course

of 65 days. Either the extracts were not potent or the insects could not respond after their period of retardation upon Diet 108.

THE FEEDING OF CONCENTRATES OF B_1 AND G

A basal diet was employed consisting of casein 40, salt mixture 4, and starch 66. To each 100 grams of this mixture were added 3 grams of cottonseed oil. Experiments were run in triplicate using bottles with ten insects in each.

TABLE 6

Diet		Mean Wt. 10 Days	Mean Wt. 1 Month	No. Alive 10 Days	No. Alive 1 Month
Basal alone.....	Group	3.6	5.0	10	8
	Group	4.2	5.0	10	9
	Group	3.8	5.0	10	9
	Individual	4.1	4.1	10	6
Basal plus 0.05....	Group	3.8	8.0	7	6
	Group	5.1	7.0	8	7
	Group	4.3	5.0	10	8
	Individual	4.5	3.8	10	7
Basal plus 0.5.....	Group	5.1	7.0	10	8
	Group	4.3	6.0	10	8
	Group	4.3	7.0	10	7
	Individual	4.9	5.2	10	7
Basal plus 5.0.....	Group	6.2	9.0	9	5
	Group	5.5	8.0	10	3
	Group	5.1	8.0	8	4
	Individual	6.0	5.2	10	7
Stock diet.....	Group	14.6	35.0	10	9
	Group	11.2	42.7	10	10
	Individual	19.0	57.5	10	10

The purpose of the first series was to add B_1 and G at two different levels and to add each separately. The sources of B_1 and G were a rice polish extract of the Burroughs, Welcome Company and "XXX liquor" of the Borden Company. Groups in these trials were weighed 10 and 20 days after the beginning as well as at the start. The results of these experiments are summarized briefly in Table 7.

These data indicate clearly that a basal diet supplemented with sources of both B_1 and G at relatively high levels gives relatively poor growth compared to a mixture of wheat and milk. This would seem to indicate that the basal diet is not satisfactory or that other vitamin fractions are essential. The best results upon the synthetic diets were obtained with a mixture of B_1 and G at relatively high levels, but these differed too little from the poorest and were too far from those fed the natural foods to be of much significance. This experiment was repeated with higher levels of the concentrate, but the results were the same and little growth resulted.

The effect of autoclaving the stock diet was now tried. The mixture of wheat flour and dry skim milk was autoclaved dry after making it into a mush. The value of the

stock diet did not seem impaired by this treatment which was made under the same conditions as those used for preparing yeast for B_1 assays.

FURTHER STUDIES WITH B FRACTIONS[1]

In order to test other B fractions a new series was now run. The composition of the diets and the fractions used as well as the results are summarized in Table 8. The G was the impure flavine from yeast (Kinnersley, O'Brien, Peters, and Reader, 1933). The "basal" diet was the mixture used at Oxford for assaying B fractions with rats. These results indicate further that yeast contains other fractions essential for this species.

TABLE 7

FAILURE OF B_1 AND G SUPPLEMENTS TO PERMIT GROWTH

Diet	Mean Wt. Begin. (Mg.)	Mean Wt. 10 Days (Mg.)	Mean Wt. 20 Days (Mg.)
Basal+0.1 per cent "B_1"+0.1 per cent "G"	1.7	2.9	3.8
Basal+0.1 per cent "G"	1.8	2.5	3.2
Basal+1.0 per cent "B_1"+1.0 per cent "G"	1.8	2.7	4.3
Basal+1 per cent "B_1"	1.7	2.9	3.5
Basal+3 per cent "G"	1.6	2.6	3.4
Wheat-milk (stock)	1.7	8.4	23.1
Basal alone	.....	2.6	3.0

TABLE 8

GROWTH WITH B_1 AND G_2 SUPPLEMENTS

Diet	Wt. Start	Wt. 20 Days	No. Alive 20 Days
Basal alone	1.06	3.1	5
Basal+B_1+G	1.06	4.9	1
Basal+B_1	1.06	3.5	7
Basal+G	1.06	1.7	2
Stock diet	1.06	9.0	8

SUPPLEMENTING BASAL DIETS WITH YEAST, WATER EXTRACT OF YEAST, AND YEAST RESIDUE AFTER WATER EXTRACTION

As an additional advance, comparisons were made between fresh yeast, its water extract, and the residue after water extraction. One hundred and fourteen grams of fresh yeast were extracted with small portions of boiling water. This was repeated six times and centrifuged between each. This yielded a total of about 60 cc. of extract. In our table of diets 5 per cent in the case of the extracts means that 5 cc. of this liquid was mixed with 100 grams of the basal diet. Duplicate groups of 14 each and of almost

[1] These studies were made in the biochemistry laboratory of Oxford University. The author wishes to express his appreciation to Professor R. Peters and to Dr. J. R. O'Brien for supplying B fractions and providing facilities for these studies.

the same mean weight at the start were used. The diets and results are summarized in Table 9.

TABLE 9

EFFECT OF FEEDING YEAST AND AQUEOUS EXTRACTS

Diet	Wt. after 4 weeks (mg.)	Diet	Wt. after 4 weeks (mg.)
Basal+10 per cent yeast (fresh)	15.6	Basal+10 per cent extract	5.8
	13.4		7.5
Basal+20 per cent yeast (fresh)	11.2	Basal+20 per cent extract	4.1
	16.6		8.6
Basal+10 per cent residue	8.6	Basal alone	2.0
	4.9		4.4
Basal+20 per cent residue	6.8	Stock diet	17.4
	4.2		13.2

These results indicated that the basal diet supplemented with fresh yeast was almost equal if not equal to the stock diet. The water extracts of yeast and the yeast residue yielded intermediate results between the basal diet alone and the yeast diets. As a whole both the residue and the extract proved poor compared to the yeast and the stock diet.

FAT SOLUBLE VITAMINS

To determine the effect of extracting the stock diet with fat solvents four experiments were run. Three groups of ten each were fed each diet:

Diet A.—Wheat-milk stock diet extracted with ethyl ether—residue only
Diet B.—Wheat-milk stock diet extracted with ethyl ether and the extract recombined with the diet
Diet C.—The same as A except petroleum ether was used
Diet D.—Diet C was recombined with the extract

Table 10 shows the weight increases for each of the groups.

TABLE 10

GROWTH UPON STOCK DIET OF WHEAT AND MILK EXTRACTED WITH ETHYL AND PETROLEUM ETHER

Diet	10 Days Mean Wt. (Mg.)	20 Days Mean Wt. (Mg.)
A. Wheat-milk-residue after extraction with Et_2O	9.4	19.7
B. Wheat-milk extracted with Et_2O and recombined with extract	10.9	29.0
C. Wheat-milk extracted with petroleum ether	8.0	18.0
D. Wheat-milk extracted with petroleum ether and recombined	10.9	30.5

The diets with the residues and the extracted materials returned were superior to the residues alone. Either some essential had been removed by the ethers or the physical condition of the diet had become unfavorable. If alcohol had preceded the ether extractions, more marked differences might have resulted. It is unlikely that either vitamin A or D is responsible for the difference although this must ultimately be checked by direct test. E may be important. Specific fatty acids and cholesterol may be essential.

ALCOHOLIC-ETHER EXTRACTION OF FOODSTUFF

In another experiment the stock diet was extracted with ethyl alcohol (95 per cent) for 48 hours. This was then followed by extraction with petroleum ether for the same time. These resulting fractions were tested in triplicate.

These data which are not included indicated that extraction with alcohol and ether removes something that is essential and which is not made good by vitamins A, D, B_1, and G. The better growth upon the stock diet indicated that the extraction and recombination may have actually destroyed some essential substances. The other possibility is that the solvents left a toxic residue

TABLE 11

LIFE SPAN OF *Blattela germanica*

Source of Insects	Diet Fed for the Remaining Life	Mean Wt. Attained in Mg.	Wt. Extremes in Mg.	Mean Life-Span in Days	Life-Span Extremes in Days
Diet 105.....	"Gelatin" basal+2 cc. Ext. (yeast) per 5 grams	6.0	4.2– 9.5	158	107–245
Diet 107.....	"Gelatin" basal+20 cc. Ext. (yeast) per 5 grams	22.2	10.4– 35.2	206	107–317
Diet 103.....	"Casein" basal+5 cc. Ext. (yeast) per 5 grams	56	21 –106	179	78–314
Diets 101, 102, 104	Casein basal+2 cc. Ext. (yeast) per 5 grams	16.4	6.4– 39.3	175	133–263

LIFE SPAN DATA

This species seems to be especially adapted for population and life span studies. It does not pass its life-cycle in the midst of its food and therefore fewer complications are introduced from excreta and bacterial action. These factors are of interest in interpreting data from insects and in drawing conclusions to assist in problems with higher animals. Individuals from the preceding assays were available for life-span studies after failure to grow owing to vitamin deficiencies.

In the present study an attempt was made to measure the life-span of individuals retarded in growth and never allowed to mature in comparison with those reared to maturity. In each case the retardation was effected by limiting the vitamin B allowed in the alcoholic extract of yeast. The individuals studied were employed originally for the assays described previously. Pertinent data regarding life-span are shown in Table 11.

These data indicate that the normal life-span for this species held at 25° C is seven months or less. These data also show that growth can be retarded for five months. In early experiments growth was resumed after retardation but no studies have been made after retardation of as large a fraction of the total life-span as five months.

SUMMARY

Methods for employing the ordinary cockroach, *germanica*, in nutrition studies have been devised. The conversion of matter into body tissue by this species indicates that

it is nearly three times as efficient as the better domestic animals such as swine. An extensive series of tests to determine the vitamin needs of this insect indicates that it must have certain essential factors found in yeast. These factors seem to be partly soluble in water and partly in lipid solvents such as alcohol and ether. These factors seem to be different from B_1 and flavine. No evidence indicates any need for fat-soluble vitamins A and D. Life-span data for this species indicate that all die within a year. Individuals whose growth has been checked by vitamin deficiencies can live for as much as five months.[2]

LITERATURE CITED

ARMSBY, H. P., and MOULTON, C. R. 1925. The animal as a converter of matter. New York.

BARRETT, J. F., and JONES, E. B. 1932. A colorimetric method for the direct determination of urea in urine. Biochem. Jour., **26,** 1246.

CROWELL, M. F., and MCCAY, C. M. 1937. Nutritional studies of the webbing clothes moth. *Tineola bisselliella* Hum. Physiol. Zoöl., **10,** 368.

KINNERSLEY, H. W.; O'BRIEN, J. R.; PETERS, R. A.; and READER, VERA. 1933. Large scale preparations of vitamin B_1 and B_4 concentrates. Biochem. Jour., **27,** 225.

LUSK, G. 1928. The science of nutrition. Philadelphia.

MCCAY, C. M. 1937. The biochemistry of fish. Ann. Rev. Biochem., **6,** 445.

———. 1933. An insect test for vitamin B fractions. Proc. Amer. Soc. Biol. Chemists **100,** lxviv.

MORRIS, H. P.; NELSON, J. W.; and PALMER, L. S. 1931. The quantitative determination of calcium, magnesium, and phosphorus in feedstuffs and excreta. Jour. Ind. and Eng. Chem., **3,** 164.

OSBORNE, T. B., and MENDEL, L. B. 1919. The nutritive value of the wheat kernel and its milling products. Jour. Biol. Chem., **37,** 557.

PALMER, L. S., and KENNEDY, C. 1931. The fundamental food requirements for the growth of the rat. Jour. Biol. Chem., **90,** 545.

TRAGER, WM. 1935. On the nutritional requirements of mosquito larvae, *Aedes Aegypti*. Amer. Jour. Hyg., **22,** 475.

WOLLMAN, E. 1926. Observation sur une lignee aseptique des Blattes (*Blattella germanica*). C. R. Soc. de Biol., **95,** 164.

YASUDA, M. 1931. The determination of the iodine number of lipids. Jour. Biol. Chem., **94,** 401.

YOUNGBERG, G., and YOUNGBERG, M. 1930. A system of blood phosphorus analysis. Jour. Lab. and Clin. Med., **16,** 158.

ZABINSKI, JAN. 1929. The growth of blackbeetles and cockroaches on artificial and on incomplete diets. Jour. Exper. Biol., **6,** 360.

[2] In the course of these studies the author is indebted for assistance to Dr. Mary Crowell, Dr. J. de Tomasi, and Mr. R. E. Bowers as well as members of the biochemistry department of Oxford University, England.